Vol. 28, No. 3 September 1951

The Journal of Experimental Biology

EDITED BY
J. GRAY

ASSISTED BY
The Hon. Secs. Society for Experimental Biology

Contents

Published for The Company of Biologists Limited

CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON

AGENT FOR U.S.A.
ACADEMIC PRESS INC.,
125, EAST 23RD STREET, NEW YORK, U.S.A.

Price 21s. net *Subscription per volume 63s. net*

THE JOURNAL OF PHYSIOLOGY

AUGUST 1951. VOL. 114, NO. 4

Subscription price 45s. per volume of 4 parts

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

EXPERIMENTS ON THE NEURAL CREST OF THE LAMPREY EMBRYO

BY D. R. NEWTH

Department of Zoology and Comparative Anatomy, University College, London

(*Received 7 November* 1950)

(With Plates 3 and 4 and Three Text-figures)

INTRODUCTION

It is characteristic of the early development of the vertebrate animals that the embryo should be formed largely as a result of successive displacements of the cell population created during and after cleavage. Of these the first bring the endoderm and the mesoderm into what are effectively their definitive positions by paths that vary somewhat from one vertebrate to another. They are followed by morphogenetic movements which see the medullary system, originally external, rendered internal; and this process is substantially constant throughout the phylum. Both by virtue of its massiveness, and by the ubiquity and variety of the tissues to which it gives rise, this system would merit the status of a fourth 'germ-layer' were it not that it is above all from the study of its neural crest component that the limitations of the germ-layer concept in vertebrate embryology has been most strikingly demonstrated (de Beer, 1947).

Nevertheless, as far as our present knowledge goes, the neural crest alone is an embryonic tissue whose fate in different vertebrate classes shows the constancy to be expected of a 'germ-layer'. Thus it certainly gives rise to the spinal ganglia, parts of the dorsal root ganglia in the head, and some of the sheath cells of the peripheral nervous system. In Amphibia it is known to form the bulk of the cartilages of the splanchnocranium and the anterior portion of the trabeculae cranii, and a similar situation probably exists in selachians (Dohrn, 1902), teleosts (Lopashov, 1944) and birds (Goronowitsch, 1892). In Amphibia, birds and teleosts it is an important, if not the sole, source of chromatophores.

For more than one reason it is of interest to know something of the fate of the neural crest in Cyclostomes. In the first place it is possible that the crest was not primitively responsible for the diverse tissues to which it gives rise in the living Gnathostomes. It might therefore be expected that the Cyclostomes would show either the primitive or an intermediate condition, the determination of which would serve as an important pointer to the evolution of the crest. The situation is complicated, however, by the uncertainty that exists on the morphological status to be given to the visceral skeleton and so-called trabeculae cranii in lamprey, the Cyclostome most readily available for embryological study. If, as Rathke (1832) and Balfour (1881) thought, the branchial basket of these animals is not comparable with the visceral skeleton of Gnathostomes, and if, as Sewertzoff (1916) and de Beer (1931, 1937)

have held, the lamprey 'trabeculae cranii' are really anterior parachordals, failure of these structures to owe their origin to the neural crest is not open to a single explanation. These points will be further considered when the results of the present investigation are discussed.

The present state of our knowledge of the fate of the neural crest in lampreys is unsatisfactory. The crest itself was identified by Koltzoff (1901), who thought that it formed part at least of the skeletogenous mesenchyme of the head. Damas (1944), in a recent thorough investigation of the development of the head, comes to the same conclusion, but would derive the 'trabeculae cranii' from ectomesenchyme and not from sclerotome as did Koltzoff. On this last point Johnels (1948) is in general agreement with Koltzoff. Only Hatta (1915) denies a crest contribution to the mesenchyme (which he nevertheless calls mesectoderm!). On the other hand, von Kupffer (1895) and Schalk (1913) claimed to trace the origin of the branchial bars from the ectoderm in the branchial region, and Damas and others agree that there is an extensive placodal contribution to the ectomesenchyme. The material is so unfavourable for normal histological methods that it is doubtful if they will ever permit a clarification of these points.

In respect of other crest derivatives we have only the isolated observations of Bytinski-Salz (1937). After homoplastic transplantation of the anterior neural cord into the blastocoele of a blastula he obtained homoiogenetic inductions of a secondary neural system. Among the induced structures there were, in two individuals, melanophores that he identified as of graft origin. Uncertain as to the possession by lampreys of a neural crest he nevertheless suggested a parallel between this result and amphibian experience.

In this paper are recorded and discussed the beginnings of an experimental approach to the problems presented by the neural crest in the lamprey.

MATERIALS AND METHODS

Eggs of the brook lamprey (*Lampetra planeri* Bloch) were used in these experiments. The early development of this species differs in no known fashion from that of the river lamprey (*L. fluviatilis*). Spawning adults taken from the nest and placed in shallow sinks in the laboratory will, after a short interval, resume nest-building activity and then continue spawning until spent. The great majority of eggs obtained from them are fertile. The culture of these eggs, if they are left in their capsules, presents no difficulties until the stage when, as young ammocoetes, their last reserves of yolk are consumed. The most serious of their requirements seems to be low temperature, for they will thrive only in water kept below 15° C.

The animals used for experiments were decapsulated with fine forceps shortly before suffering operation. This procedure is not difficult at the late neurula stage concerned; about 15% of all animals were damaged and discarded before operation. The naked embryo, until immediately before the age at which it would normally have hatched, is very delicate and can be mortally injured by lengthy contact with glass surfaces. For this reason decapsulation, operation and subsequent culture took place in vessels lined with an agar-agar gel. No specific culture fluid for lamprey

embryos has been developed, and so, following the example of Bytinski-Salz, full-strength Holtfreter solution was used for embryos from decapsulation until about 1 hr. after operation. They were then transferred to Holtfreter solution diluted ten times. In this they were kept for 3 days, after which they were transferred to water. All fluids bathing naked embryos contained 1·0 g. of sulphadiazine sodium for every 500 ml.

The operations were performed with the aid of knives ground from steel needles, loops of baby's hair and glass bridges. They involved no technical novelty and need not be described in detail. Post-operative mortality was high mainly because a successful experiment required so long a period between operation and killing for histological study. Mortality is detailed and discussed with the other results.

Animals required for histological examination were killed in Smith's fluid embedded after pre-impregnation with celloidin by the method of Peterfi, and sectioned at 5 or 6 μ. This treatment, followed by staining with Ehrlich's haematoxylin and eosin, gave admirable results for animals of all ages between neurula and young ammocoetes.

RESULTS

(1) *Defect experiments*

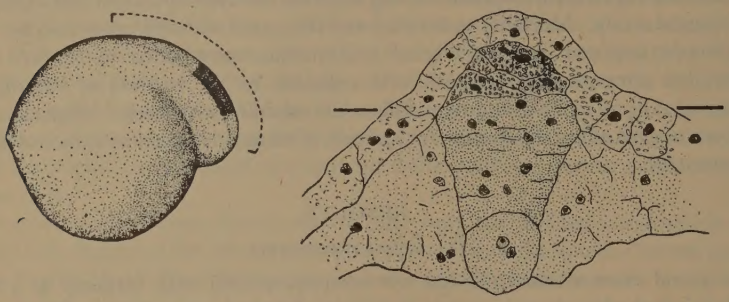
The neural crest in lampreys has not been recognized with certainty at a stage earlier than the late neurula, in which it lies mid-dorsally as an unpaired strip above the medullary cord proper. This, then, represents the earliest stage at which operative procedures on the crest may be practised with confidence. It is, furthermore, impossible to identify the crest cells in dissected embryos (it is difficult enough in sections) after their ventral migration has begun. The short period in which these cells are concentrated in the mid-dorsal line thus determines the stage at which experiments are performed. The advantages offered by amphibian material where the crest may be attacked at any stage between an early neurula and one in which migration is well advanced are here lacking. This limitation is serious in transplantation experiments, but is of less importance for the defect experiments now to be described.

The purpose of these experiments was to determine the derivatives of the neural crest in the head region by examination of young larvae from which parts of that tissue had been removed at an earlier stage. Experiments were performed in two successive years, the results of the first year have been briefly reported in Newth (1950). The two series were treated in a similar way and will be considered together.

The tissue removed in a typical experiment is shown in Text-fig. 1. In so small and pigment-free an embryo the precise extent of the removed mass cannot be judged with certainty. I have, however, satisfied myself by examining embryos killed immediately after operation that the crest was effectively extirpated in the region intended. Indeed, frequently somewhat more of the medullary cord than is indicated in Text-fig. 1 went with it. In order to avoid too great a strain upon any one embryo, no less than to obtain information on the fates of different regions of the head crest, a limited part of the crest was taken in each experiment.

About 120 operations were made, but of these only thirty were successful in the sense that the animals survived sufficiently long to make histological examination worth while. Of these twenty-one were old enough at death to possess differentiated melanophores and recognizable cartilage or pro-cartilage in the visceral arches and at the site of the 'trabeculae cranii'. The other nine had all reached an age at which their cranial ganglia should have been discrete and recognizable.

The post-operative development of the successful experimental animals appeared to be quite normal. The wound was completely healed within 24 hr. in all but one case; in this one the wound area remained recognizable until death as a depression



Text-fig. 1. The defect experiments. This tissue removed in a typical experiment is darkly shaded on the left-hand figure. The region covered in the experimental series is indicated by the dotted line. On the right a transverse section of an embryo of the same age is drawn to show the disposition of the tissues in the dorsal part of the animal. The neural crest is here shaded darkly. Pl. 4, fig. 5, is a photomicrograph of part of a similar section.

in the dorsal surface of the animal. In some animals the depth of pigmentation on the head seemed to vary from the normal, in four cases it appeared to be less deep, in three others deeper than in control animals. To what extent this is significant will be discussed below. The results of the histological examination of the thirty animals are best analysed with reference to the tissues suspected of crest origin.

The nervous system. Sections of the experimental animals showed an overall normality. Thus in all cases the ectoderm of the dorsal part of the head had healed completely and no trace of scar remained. More importantly the brain was always complete and normal in appearance, clearly as a result of regulative processes in the period immediately following operation. Eyes and otic vesicles were unaffected by the experiment.

It was to be expected that the cranial ganglia of the dorsal series (V_1, V_2, VII, IX and X) might show themselves to be derived in part from the neural crest by deficiencies in the experimental animals. In fact all of these ganglia were affected in one or other animal, the deficiencies, either unilateral or bilateral, being sometimes of a surprising extent. Thus in one animal the vagus ganglion on one side was almost completely absent, though its partner was only partly affected. The section through this animal illustrated in Pl. 3, fig. 4*c* has been chosen to include the few isolated nerve cells that take the place of the missing ganglion. Normal and experimental

studies of members of other vertebrate classes have accustomed us to the fact that the respective contributions of crest and placodes to the head ganglia varies considerably, but this result remains extraordinary. The animal is generally normal, and it would appear that one of two explanations of its condition must be accepted. Either the lamprey vagus is derived almost exclusively from the neural crest, or the successful organization of its placodal component is dependent upon the presence of the crest. The second alternative, though out of keeping with amphibian results, seems the more likely. Certainly there is no reason to believe that the vagus in this species lacks fibres generally thought to be of placodal origin (lateralis), and Damas (1944) has described convincingly the process by which placode cells are normally added to the vagal primordium. This result undoubtedly invites further investigation.

In no other case was the reduction in a ganglion so great, though the facialis, glossopharyngeus and vagus were all sometimes very small. Extreme examples of reduction are shown in Pl. 3 with sections through control animals for comparison. The trigeminus was never found to be less than two-thirds of its normal bulk, and if anything the profundus was affected to a still lesser degree. It is difficult to decide with certainty whether a ganglion is affected at all unless the reduction is either very great or else bilaterally asymmetrical. Comparison between control and experimental animals cannot be quantitatively accurate unless the stage of development of each precisely corresponds, and this is not easy to establish. However, I am satisfied that clear defects were to be found in the profundus four times (of which three were bilateral), in the trigeminus twice (both bilateral), in the facialis five times (none bilateral), in the glossopharyngeus five times (two bilateral), and the vagus nine times (six bilateral). The first spinal dorsal root ganglion was affected in three cases. In nine animals no certain defects were observed, and in them effective regulation of the crest must be assumed to have occurred.

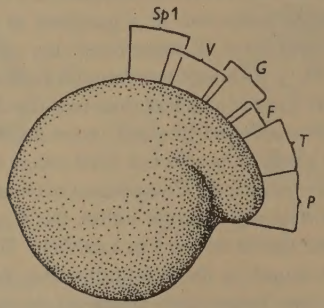
The correlation between level of operation on the neurula and subsequent defect was sufficiently good to enable a provisional mapping of the neural crest in terms of its fate. Text-fig. 2 shows the result. It appears from this that the position of the crest components of the cranial ganglia corresponds well with the position of the mesodermal segments with which they will later be associated (for the latter see Damas, 1944). My operations were not, however, sufficiently refined to confirm Damas's observation of a short region above the hyoid segment devoid of neural crest.

Cartilage. Those experimental animals reaching an age at which the first formed cartilages of the branchial basket would normally be present were killed for examination to avoid the risk of their loss from casual death and histolysis. At this stage (6·8 mm. in length, approximately 25 days old) the young animal has chondrified vertical bars with rudimentary epi- and hypotrematic elements in the visceral arches, and well-developed pro-cartilaginous rudiments of the 'trabeculae cranii'.

In no single case was neural crest removal observed to result in deficiencies in these structures.

This result can be explained in several ways. The simplest is to assume that there is no morphogenetic relationship between the crest and the cartilaginous head

skeleton, but before this assumption is accepted three alternative explanations must be considered. First, it is possible that skeletal structures not yet apparent in animals of this age might have been affected. This is not, in my opinion, a serious objection in the case of the incomplete branchial basket whose morphological integrity is hardly to be doubted, but has some force when applied to the 'trabeculae'. Johnels (1948), who believes that these are primarily somitic in origin, does not exclude the possibility that their anterior parts—in particular the trabecular commissure—are derived from ectomesenchyme. The results of my experiments do not help here, since it is only in older animals that the commissure is formed.



Text-fig. 2. The fate of different regions of the anterior neural crest of the neurula as shown by defect experiments. The approximate extent of the primordia of the ganglia are indicated. *P*, profundus; *T*, trigeminus; *F*, facialis; *G*, glossopharyngeus; *V*, vagus; *Sp.* 1, first spinal dorsal root ganglion.

The second alternative is that effective regulation of the crest has made good the deficiencies in skeletogenous material. This is, of course, quite possible, particularly if considered in conjunction with the third objection, namely, that the high mortality in experimental animals was selective, removing those which had suffered the most radical operations, and hence the most complete removal of crest tissue. Had there been even one animal which showed the slightest deficiency in its head skeleton this possibility would certainly have to be invoked to account for the others which did not. As it is, however, the absence of skeletal defects when considered together with the effects on the neural derivatives of the crest makes it unlikely that this is the proper explanation.

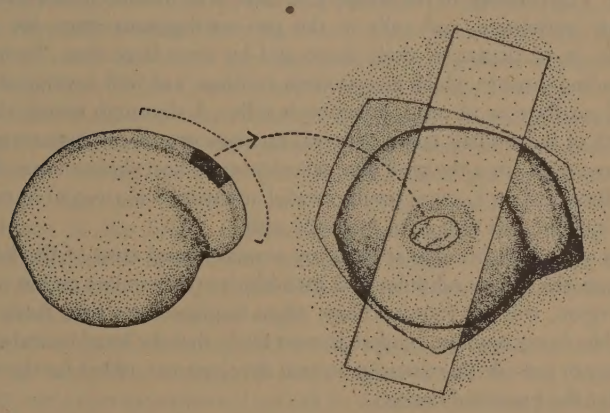
The defect experiments cannot be said to prove the non-crest origin of the lamprey visceral skeleton, but it is fair to conclude from them that it is unlikely that either the branchial basket or the major part of the 'trabeculae cranii' are formed from the neural crest. This conclusion, it must be stressed, does not apply to the larval muco-cartilage.

Melanophores. It has already been mentioned that a few of the experimental animals showed signs of a disturbance of their pigmentation while still alive. This impression was confirmed in study of sections, but it cannot be said that the defect

experiments provided satisfactory proof of a relationship between crest and melanophore numbers. In the three animals in which crest removal appeared to lead to an increase in melanophore number the effect was, however, sufficiently striking to be worthy of notice. If later work should show these observations to be trustworthy the explanation is probably to be found in the interesting results of Niu (1947). He found that if the *whole* of the head crest was removed from Urodele embryos the remaining neural tube over-compensated for the loss by providing many more melanophores than were present in normal animals. Lopashov (1944) also had reason to believe that melanophores arise in the head of teleosts from all parts of the medullary cord.

(2) *Transplantation experiments*

Living crest cells homoplastically transplanted may be expected, in certain circumstances, to give rise to tissues corresponding to their normal fate. In a series of experiments designed to exploit this situation pieces of head crest were grafted



Text-fig. 3. The transplantation experiments. The figure of the donor on the left shows the extent of the tissues grafted in a typical experiment and the region from which similar grafts were taken in the experimental series. The figure of the host on the right shows the graft site and method of grafting.

into the flank of other embryos. The grafts consisted of pieces that precisely corresponded to those removed in the defect experiments, and they therefore always contained donor ectoderm, and usually some of the presumptive brain, as well as neural crest (see Text-fig. 3). Host animals were chosen to be a very little younger than donors so that if, after killing, host tissues of suspected crest origin (cartilage, etc.) were seen to be present, time alone should not prevent similar differentiations in cells of graft derivation. Grafts were taken from all regions of the head crest.

In twenty of the twenty-eight experiments made the grafts healed in well, but in the remaining eight they were extruded soon after operation. Of the twenty good hosts eleven survived until an age at which normal cartilage or recognizable pro-

cartilage of host origin was present in the head region. Some of the other nine have, however, provided information of interest to another problem.

The site of the graft usually remained identifiable in the living animal as a small bulge in the ectoderm. The first indication of morphogenetic activity on the part of the implanted neural crest was the appearance in its neighbourhood of differentiating melanophores. These increased in numbers and came to cover a wider area of the flank as time passed. Their first appearance coincided approximately with that of the host's melanophores in the head region, that is to say, considerably before the appearance of host melanophores in the trunk. They thus behave 'herkunftsgemäss' in respect of rate of development.

Sections showed that the grafts in all cases lay immediately below the ectoderm, between it and the somatopleur. In every animal the graft had given rise to a mass of nervous tissue, sometimes solid and sometimes with a lumen (Pl. 4, figs. 7, 8, 9, 10 and 11). The results of histological examination will be considered under separate headings.

Cartilage. The cartilage of the lamprey embryo is as distinct histologically as that of any other vertebrate, and cells in the pro-cartilaginous stage are also easily recognizable, in particular by their shape and by their large size. In none of the grafts, including those in which host visceral cartilage was well developed, was there any sign of cartilage or of pro-cartilaginous cells. A thorough search through the whole length of the animals failed to reveal cartilage or pro-cartilage except in those places, far removed from the graft site, where normal host skeleton was developing. In view of Raven's (1933, 1935) results careful attention was given to internal organs (liver, gut wall, etc.) as well as to the body wall.

It is thus very probable that in lamprey cranial neural crest of the late neurula, whether from the pre-mandibular and mandibular (trabecular) region or from the branchial region, will not form cartilage when implanted into the flank of another embryo. This, in my opinion, renders it most likely that the head neural crest in this species does not provide this tissue in normal development, either for the 'trabeculae cranii' or for the branchial basket.

These experiments are open to one important criticism in the light of the lessons we have learned from the Amphibia. Contrary to the views earlier expressed by Stone (1926), Raven (1933, 1935), and Ichikawa (1937), it now seems certain that the amphibian crest (or at least that of Urodeles) is not 'determined' to cartilage formation in the early neurula. Hörstadius & Sellman (1945) have convincingly demonstrated this point and have suggested a reasonable explanation of the work of other authors that appears to contradict it. It is thus necessary to consider whether the failure of the lamprey crest to develop into cartilage in the conditions of these experiments is due to its determination in normal development at a stage after that at which it was transplanted, e.g. in response to contact with the branchial endoderm. Three points may be made in this connexion. First the operative technique used certainly involved just such damage to underlying host tissues as Hörstadius & Sellman consider provokes cartilage formation in crest grafts to the flank in Amphibia. Secondly, the crest from the donor in the lamprey experiments, although

not yet in the migratory phase, is, nevertheless, 'older' morphogenetically than that of the open neural plate stages used by these authors. Lastly, and most importantly, Lopashov (1944) has found that in teleosts transplanted neural tissue from the head will give rise to cartilage when transplanted to the yolk-sac or flank of another embryo. His grafts were taken from donors with a solid neural cord and a mid-dorsal neural crest, i.e. comparable to the lamprey donors in point of morphogenetic age.

These considerations do not completely eliminate the doubts to which the results of Hörstadius & Sellman must give rise, but they do, in my opinion, enable us to say that it is extremely unlikely that the cranial neural crest in lamprey is skeletogenous. Taken in conjunction with the results of the defect experiments the matter seems to me to be hardly in doubt. If grafts of head crest to the head region of other embryos also give negative results the demonstration will be complete. This test I hope to apply in the coming breeding season.

Mesenchyme and chromatophores. Sections revealed isolated cells in the neighbourhood of each graft lying between the lateral plate mesoderm and the ectoderm of the host. In the younger animals it was not possible to identify them from their appearance, they might equally have been neuroblasts or mesenchyme cells. In the older ones they could be seen to be mesenchymatous. In none of the experiments were they very numerous, but some could always be found. That they originated in the graft is most likely as sections through comparable regions of control animals failed to show similar cells. This serves to confirm Damas's observations, based on serial sections of normal material, that the head crest has an ectomesenchymal component. It does not, however, mean that the crest is the sole source of ectomesenchyme in the head. On the contrary, Damas has shown that both placodes and stomodaeal invagination contribute largely to this tissue, and it may well be that the diverse cell types that differentiate from the ectomesenchyme are strictly related to its several sites of origin.

The differentiation of melanophores in the region surrounding the graft is of interest from several points of view. In the first place it must be said that in the light of amphibian and avian experience it cannot be doubted that they are of graft origin. They thus provide a good marker of the migratory capacity of crest cells. Damas has, indeed, described this migration from his sections, but the difficulties in interpreting sections of young lamprey material makes direct confirmation of value. The extent of the migration from the graft site can be seen in Pl. 4, fig. 6, which shows that the distance over which crest cells can migrate is quite as large as that of their amphibian analogues.

As with the mesenchyme, however, it must not be assumed that the crest is necessarily the sole source of melanophores in normal development. Lopashov (1944) has found indications that in teleosts the whole brain may provide them, and this may be true of lower vertebrates generally, while Niu (1947) has shown that the brain will do so in Amphibia under experimental conditions. The positive result of these transplantation experiments as far as the melanophores are concerned has as its most important consequence the demonstration that a migratory neural crest with components destined to a non-neural fate is a property of craniate vertebrates, both

jawed and jawless. It is thus almost certainly an archaic feature characteristic of the common ancestors of the Agnatha and Gnathostomata.

The lumen of the neural canal. Holtfreter (1933, 1934) has shown that in Amphibia the disposition of the cell bodies in the neural tube, and the shape and position of its lumen, are determined by the nature of its environment. Thus an isolated embryonic neural 'tube' is, in fact, solid with the cell bodies at its periphery; one surrounded by mesenchyme has a central lumen, circular in cross-section, with the cell bodies centripetally arranged; one in contact with a block of somitic muscle has its lumen displaced to the opposite side; while one in contact with a chorda has its lumen elongated in cross-section with the long axis pointing towards the contiguous tissue. It is easy to see that these results explain the form of the normal spinal cord in terms of its relations with the flanking myotomes and the underlying notochord.

Huxley & de Beer (1934) have suggested that these results may shed light on the evolution of the vertebrate brain, in the sense that if they were generally valid an environment of myotomes (as is suffered by the anterior neural tube of *Amphioxus*) would militate against the formation of a vesicular, thin-walled brain. It is therefore of interest to observe that in the lamprey those grafts which differentiated into neural masses with a lumen had the form that Holtfreter's results would have led one to expect (Pl. 4, figs. 7, 10 and 11). In them the wall of the neural vesicle which lies against the lateral plate mesoderm is always thick, while that lying against mesenchyme or ectoderm is thin. In one case (Pl. 4, fig. 11), in which the vesicle had two opposed walls in contact with the ectoderm, a structure closely resembling the normal neural tube resulted. It thus appears that endodermal tissues may exercise an effect similar to that of musculature in this process. The grafts which remained solid are not relevant to this problem as they were undoubtedly ganglionic in nature, having few or no cells from the brain or spinal cord proper (Pl. 4, figs. 8, 9). We are again forced to admit that a developmental process common to both Amphibia and lamprey was probably shared by their ancient common ancestral stock. To this extent the value of the suggestion of Huxley & de Beer is enhanced.

DISCUSSION

That the possession of a migratory neural crest with components destined to non-neural fates is a feature not only of Gnathostomes but of all the craniate chordates is not surprising. The establishment of the capacity of the lamprey neural crest to form melanophores assumes additional importance, however, when we come to consider its apparent incapacity to form cartilage. This failure cannot be associated with lack of opportunity, in the sense that crest cells migrate to regions in which visceral cartilages are formed, the histogenesis of cartilage and the formation of melanophores in the branchial arches taking place contiguously and almost simultaneously. The actual origin of the visceral skeleton in lamprey cannot yet be stated with certainty, but the admirable observations of Damas (1944) command attention. He finds that the branchial bars (and also the 'trabeculae cranii') are indeed formed from ectomesenchyme, but that this tissue has a composite origin being derived both from the crest and from an extensive placodal contribution. It

thus seems that the earlier conceptions of von Kupffer (1895) and Schalk (1913) may well prove to be correct. They found that the branchial skeleton was formed from cells given off from the deeper layers of the overlying ectoderm (branchiodermis of Schalk). It is almost certain that Hatta's (1915) belief in the mesodermal origin of these cartilages is ill-founded.

As to the 'trabeculae cranii' Damas's observations have not been confirmed by Johnels (1948), who believes, as did Koltzoff (1901), that they are formed from the sclerotome of the anterior head somites. The present work is, of course, quite consistent with this belief.

Now if it be true that the 'trabeculae cranii' of lamprey are derived from sclerotome, and that the branchial basket is not of neural crest origin, the homologies of these structures with their gnathostome analogues is called into question. It must, of course, be recognized that embryological origin cannot be the sole criterion of homology, and that it is possible to accept the homology of elements of the lamprey and gnathostome splanchnocranium without reference to embryology. It so happens, however, that these homologies have for some time been suspect on morphological grounds. We may therefore fairly cite the embryological evidence against them without thereby claiming that it is alone sufficient to determine the issue.

The evolution of the gnathostome visceral skeleton is now well established in a general sense from the Aphetohyoidean grade of organization onwards. The discovery that the anterior portion of the trabeculae cranii in Amphibia share the neural crest origin of the cartilages of the jaws and visceral bars adds strong support to the view of Allis (1923) and de Beer (1931) that the trabeculae represent skeletal elements from a pre-mandibular visceral arch.* It is thus to be considered probable that there existed pre-Aphetohyoidean gnathostome ancestors which possessed one functional gill slit before the spiracle. The backward growth of the mouth must, of necessity, have obliterated this slit, while the skeletal element lying in the pre-mandibular arch (or part of it) then came to serve the needs of the neurocranium as a trabecula. Holmgren (1949) has put forward objections to this view on the basis of his studies of the origin of the trabeculae in fishes, but the balance of considerations favours it.

It follows from this that if the lampreys possess true trabeculae cranii they, or their ancestors, must possess or have possessed a series of gill bars of the same nature as those of the Gnathostomes, i.e. homologous with them. Certainly the lamprey condition in which the first functional gill opening is post-spiracular is secondary, for, as Stensiö (1927) has shown, the Osteostraci are not only the most likely lamprey ancestors, but also possessed two more anterior openings. Thus lamprey ancestors may be assumed to have had a functional pre-mandibular arch between the mouth and the first gill-opening. But is the agnathan visceral skeleton to be homologized with that of the Gnathostomes?

* Serial homology is clearly a reality though no more to be considered as a rigid system than normal homology. In particular the weight to be given to embryological origin in determining serial homology must be separately estimated in every case. The experiments of Bijtel (1931) and Ford (1950) show that such serially homologous structures as myotomes may have diverse anlagen in a single individual.

Rathke (1832) first opposed the 'external' branchial basket of lampreys to the 'internal' gill bars of Gnathostomes. In this he has been followed by some later workers, notably Balfour (1881). Others (Dohrn, 1902; Balabai, 1937) have sought to homologize them. The literature on this subject is too extensive for review here, but it must be mentioned that in two cases it has been suggested that elements of the non-prevailing type of visceral skeleton can be found in living forms. Thus it has long been thought that the extra-branchialia that occur in some selachians were parts of an external arch system, and Holmgren (1946) has recently discovered structures in a *Myxine* embryo which he regards as elements of the internal arch skeleton of the mandibular, hyoid and first branchial segments. These results of Holmgren are of the greatest interest, but, unfortunately, only serve to emphasize the need for a thorough knowledge of progressive stages in the development of *Myxine*.

While the morphological status of both the extra-branchialia and Holmgren's internal arches in *Myxine* cannot yet be regarded as established, the more general statement of the non-equivalence of the major elements in the agnathous and gnathostome visceral skeleton commands respect. Of the two the cyclostome splanchnocranium is the more easily interpreted. Following Stensiö (1927) and Holmgren & Stensiö (1936) we may regard the lamprey branchial basket (and probably the muco-cartilaginous elements in the Ammocoetes) as a remnant of the originally continuous endoskeleton of the Osteostraci. Damas (1944) has gone further in suggesting that the distribution of the ectomesenchyme in lamprey embryos before parts of it have given rise to cartilage or muco-cartilage is such as to permit comparison between this embryonic tissue and the endoskeleton of the adult Ostracoderm. Be this as it may, there is no reason to suppose that lampreys ever had a system of discrete bars as a gill skeleton. The similarities in gill function between *Cephalaspis* and lamprey make it quite reasonable to assume that the visceral skeleton of the latter is derived from that of the former by the simple loss of most of the primitively continuous structure. The fact that the first elements of the branchial basket to chondrify are the vertical rods does not at all justify their comparison with the bars of Gnathostomes made by Sewertzoff (1916).

Now this interpretation does not preclude there being present in the lamprey some equivalent of the skeleton of its ancestor's pre-oral arches. Sewertzoff (1916) even claimed to find elements of three pre-mandibular arches, but this is so at variance with all our present knowledge of the anterior region of the craniate head that it need not be considered further. Of the workers who have studied this problem in recent years none has suggested that the 'trabeculae cranii' are pre-mandibular arch elements. Indeed, the evolution of the Agnathan head has not been accompanied by that backward growth of the mouth typical of Gnathostomes, and consequently there is less reason for supposing that visceral elements in the pre-mandibular region should be 'pushed' upwards to become attached to the neurocranium as trabeculae. There is, in fact, no reason at all for believing the 'trabeculae' of lampreys to be visceral.

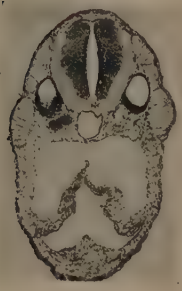
When we come to the Gnathostomes difficulties arise. It is almost certain that the osteostracan pre-spiracular gill opening is a primitive craniate feature, the loss of anterior gill openings in Agnatha and Gnathostomes being an example of major

a

b

Fig. 1.

c



a

b

Fig. 2.

c

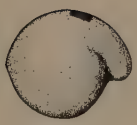
a

b

Fig. 3.

c

a



b

Fig. 4.

c

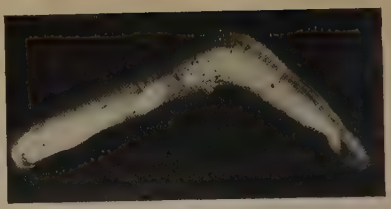


Fig. 6.

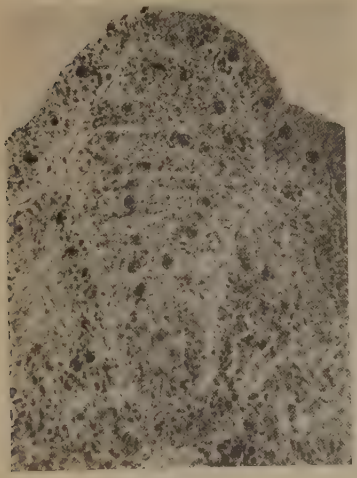


Fig. 5.

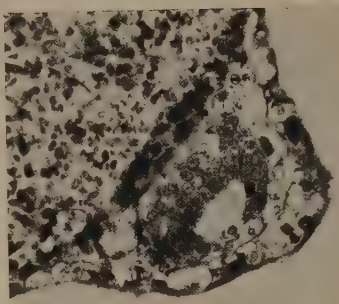


Fig. 7.

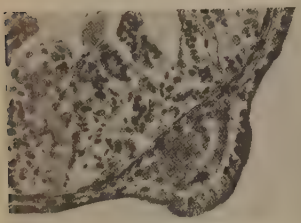


Fig. 8.

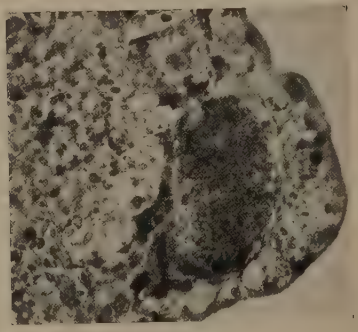


Fig. 9.

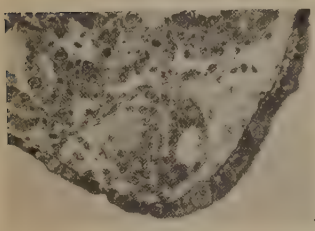


Fig. 10.

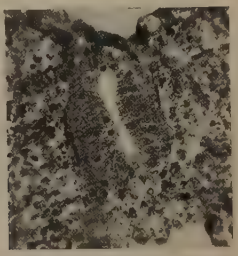


Fig. 11.

parallel evolution. This being so, are Osteostraci possible Gnathostome ancestors? If the interpretation of the trabeculae as visceral elements of an internal pre-mandibular arch skeleton is correct this seems unlikely. Rather should we expect the Gnathostome ancestor of the grade possessing a pre-spiracular gill opening to have a gill system of the slit type with a visceral skeleton composed of bars not wholly unlike the branchial bars of living fish. In the absence of fossil evidence it would be wrong to assert that this was so. All that can be said is that the present state of our embryological knowledge supports this conception, but that the much-to-be-desired knowledge of the development of *Myxine* may yet clarify these points in some different sense. In particular, it must be borne in mind that the visceral endoskeleton of the ostracoderms may have two distinct descendants: the dorsal endocranium becoming the lamprey branchial basket, and the ventral the gnathostome system of bars.

SUMMARY

1. The fate of the neural crest of the head in *Lampetra planeri* has been studied by experiments involving the removal or the homoplastic transplantation of short regions of this tissue.

2. The results show that the neural crest of lamprey is normally destined to form part at least of the dorsal root ganglia, and that it probably gives rise to most, if not all, of the melanophores and to some of the ectomesenchyme.

3. No evidence that the neural crest of the head was the source of the 'trabeculae cranii' or the cartilages of the branchial basket was found. It is concluded that these structures are most probably not crest derivatives.

4. The phylogenetic significance of the embryology of the lamprey 'trabeculae cranii' and visceral skeleton is discussed.

5. The transplantation experiments provided some evidence that the form of the neural tube in lamprey is determined in the same manner as that established by Holtfreter (1933, 1934) for Amphibia.

I have to thank Prof. D. M. S. Watson for his encouragement and interest, Dr G. R. de Beer, Mr M. Abercrombie and Dr P. Ford for advice, and Mr A. R. Hockley for his generous help in providing spawning lampreys. I am indebted to Mrs S. M. McCarthy and Mr W. Brackenbury for technical assistance.

REFERENCES

Allis, E. P. (1923). *J. Anat., Lond.*, **58**, 37.
Balabai, P. P. (1937). *Trav. Morph. Animaux Kiev*, **4**.
Balfour, F. M. (1881). *A Treatise on Comparative Embryology*. London.
de Beer, G. R. (1931). *Quart. J. Micr. Sci.* **74**, 701.
de Beer, G. R. (1937). *The Development of the Vertebrate Skull*. Oxford University Press.
de Beer, G. R. (1947). *Proc. Roy. Soc.* B, **134**, 377.
Bijtel, J. H. (1931). *Arch. EntwMech. Org.* **125**, 448.
Bytinski-Salz, H. (1937). *Arch. ital. Anat. Embryol.* **39**, 177.
Damas, H. (1944). *Arch. Biol., Paris*, **55**, 1.
Dohrn, A. (1902). *Mitt. zool. Sta. Neapel*, **15**, 555.
Ford, P. (1950). *Proc. Zool. Soc. Lond.* **119**, 609.
Goronowitsch, N. (1892). *Anat. Anz.* **7**, 454.

HATTA, S. (1915). *Proc. Roy. Soc.* B, **88**, 457.
HOLMGREN, N. (1946). *Acta Zool.* **27**, 1.
HOLMGREN, N. (1949). *Int. Zool. Cong. Paris*, p. 288.
HOLMGREN, N. & STENSIÖ, E. A. (1936). *Kranium und Visceralskelett der Akranier Cyclostomen und Fische.* Berlin.
HOLTFRETER, J. (1933). *Arch. EntwMech. Org.* **127**, 619.
HOLTFRETER, J. (1934). *Arch. exp. Zellforsch.* **15**, 281.
HÖRSTADIUS, S. & SELLMAN, S. (1945). *Nova Acta Soc. Sci. upsal.* ser. 4, **13**, 1.
HUXLEY, J. S. & DE BEER, G. R. (1934). *The Elements of Experimental Embryology.* Cambridge University Press.
ICHIKAWA, M. (1937). *Mem. Coll. Sci. Kyoto*, **12**, 311.
JOHNELS, A. G. (1948). *Acta Zool.* **29**, 139.
KOLTZOFF, N. K. (1901). *Bull. Soc. Nat. Mosc.* **15**, 259.
KUPFFER, C. VON (1895). *Verh. Anat. Ges.* **9**, 105.
LOPASHOV, G. V. (1944). *C.R. Acad. Sci. U.R.S.S.* **44**, 169.
NEWTH, D. R. (1950). *Nature, Lond.*, **165**, 284.
NIU, M. C. (1947). *J. Exp. Zool.* **105**, 79.
RATHKE, H. (1832). *Anatomisch-philosophische Untersuchungen, etc.* Riga.
RAVEN, CHR. P. (1933). *Arch. EntwMech. Org.* **129**, 179.
RAVEN, CHR. P. (1935). *Arch. EntwMech. Org.* **132**, 509.
SCHALK, A. (1913). *Arch. mikr. Anat.* **83**, 43.
SEWERTZOFF, A. N. (1916). *Russk. Arkh. Anat.* **1**.
STENSIÖ, E. A. (1937). *Skr. Svalbard Nordishavet, Oslo*, **12**.
STONE, L. S. (1926). *J. Exp. Zool.* **44**, 95.

EXPLANATION OF PLATES

PLATE 3

The four defect experiments here illustrated are chosen to show the most extreme reduction in the cranial ganglia V_1, VII, IX and X. On the right are shown sections through a control animal 7·0 mm. in length, in the middle are shown the operations, and on the left the resulting deficiencies.

Fig. 1. *a*, defective profundus ganglion in experimental animal. The section is not quite transverse, only the left side of the animal (right in picture) should be compared with control. *b*, operation. *c*, the profundus, lying above the eye, in a normal animal.

Fig. 2. *a*, defective posterior lobe of facialis ganglion on left side (right in picture). *b*, operation. *c*, the normal appearance of the facialis ganglion.

Fig. 3. *a*, defect in glossopharyngeus ganglion in experimental animal. The section is not quite transverse, only the left side of the animal (right in picture) should be compared with control. *b*, operation. *c*, the normal appearance of the glossopharyngeus ganglion.

Fig. 4. *a*, the vagus region of an experimental animal. Note reduction in animal's left ganglion and the almost complete absence of the right-hand one. *b*, operation. *c*, control animal in the region of the vagus ganglion.

PLATE 4

Fig. 5. The head neural crest as seen in a transverse section through a neurula (cf. Text-fig. 1).

Fig. 6. Homoplastic graft of head neural crest into flank at the neurula stage has resulted in development of melanophores in the graft region of the host. The graft can be seen as a small protruberance lying ventrally.

Fig. 7. Neural vesicle of graft origin seen in section. It is flanked by host lateral plate mesoderm on one side and by mesenchyme and ectoderm on the other.

Fig. 8. Neural mass of graft origin seen in section. It has no lumen. One or two ectomesenchymal cells of graft origin can be seen on either side of the neural tissue.

Fig. 9. Neural mass of graft origin seen in section. It has no lumen. The dark cells at its lower edge are melanophores.

Fig. 10. Neural vesicle of graft origin. Ectomesenchyme cells are present between the host lateral plate and the ectoderm.

Fig. 11. Neural vesicle of graft origin. It is flanked by host endoderm and is in contact with the ectoderm above and below.

THE REACTIONS OF THE MINNOW, *PHOXINUS PHOXINUS* (L.), TO SOLUTIONS OF PHENOL, ORTHO-CRESOL AND PARA-CRESOL

By J. R. ERICHSEN JONES

Department of Zoology, University College of Wales, Aberystwyth

(*Received* 10 *November* 1950)

(With Five Text-figures)

I. INTRODUCTION

Phenolic substances form an important type of polluting chemical in industrial wastes; they are present in certain sheep dips, in coal tar and synthetic tar and in effluents from gas works, coke ovens and factories producing disinfectants. Shelford (1917) studied the effect of phenol on fishes and found that 0·1% solutions were quickly fatal; below this concentration the toxicity fell off rapidly. Ortho-cresol was rather more toxic than phenol; para-cresol and meta-cresol less toxic. Shelford also tested the reactions of fishes to these substances in the gradient tank and concluded that they were generally 'indifferent' or 'positive' to the solutions. Mason-Jones (1930) investigated the toxicity to fish of a wide range of substances found in tar, using perch, yearling trout and trout fry and describes the symptoms produced by phenol and the cresols. At the higher concentrations there is a very characteristic rapid loss of the sense of balance; the fish swims with a wild, dashing movement and turns on its side; the gill covers, at first widely opened, are tightly closed; the respiratory movements become irregular and feeble, and before dying the fish may turn turtle. After death in strong solutions of phenol the gill tissues may be collapsed, but this symptom is not evident in the case of the cresols. Alexander, Southgate & Bassindale (1935, pp. 107–8) measured the toxicity of para-cresol and phenol to rainbow trout and also observed this rapid effect on the equilibrium of the animal, concluding that their experiments confirmed the opinion of other workers that phenolic substances act as specific poisons on the nervous system of fish. Ellis (1937, pp. 416–17) tested the toxicity of phenol to the goldfish and also states that it produces a paralysis of neuromuscular mechanisms. A detailed discussion of the pharmacology of phenol and the cresols is given by Edmunds & Gunn (1936, pp. 734–40); their action on fishes is not discussed but it is stated that in the frog phenol causes fibrillary twitching in the muscles followed by tonic convulsions and then a complete paralysis of the central nervous system.

In papers recently published in this *Journal* the writer (Jones, 1947, 1948) has described an apparatus for testing the reactions of fish to toxic solutions; this differs from the Shelford gradient tank in that water and solution are sharply differentiated so that the fish is presented with a definite concentration step, not a gradient. The

substances tested included salts of lead, zinc, copper and mercury, hydrogen sulphide and ammonia. The present paper describes similar experiments with phenol, para-cresol and ortho-cresol.

II. APPARATUS AND METHOD

The apparatus was essentially similar to that used for the experiments with sulphide solutions (Jones, 1948, pp. 23–4). One 10 l. aspirator with inflow and outflow supplied water to the right or left side of the experiment tube, and solution was supplied to left or right from a 15 l. aspirator fitted with an air intake tube. All solutions were made up immediately before use with tap water from the same supply as that connected to the water-supply aspirator, and the arrangement for automatically making up fresh solution which was used for the sulphide solutions was not necessary. The tube in which the fish were placed was a little larger than that used in the previous studies, measuring 34 mm. in internal diameter; this permitted the use of fish 28–32 mm. in length. With this larger tube a little difficulty was experienced in obtaining a sharp, vertical separation of water and solution in the centre; this was overcome by fitting small glass baffles in front of the internal apertures of the inlet tubes.

The method of recording the results follows that devised by Shelford (1917). The space between two parallel lines represents the length of the tube, minutes are marked off on a descending vertical scale and the movements of the fish are copied. In all the experiments only one fish was placed in the tube; as one of the chief symptoms in the case of phenolic substances is very rapid and erratic swimming, the use of more than one is impracticable.

III. PHENOL, C_6H_5OH

Survival curves for 25–30 mm. minnows in phenol, para-cresol and ortho-cresol solutions are drawn in Fig. 1. The concentration is expressed in percentage (g. phenol, etc., per 100 ml. solution). On this basis phenol is distinctly the most toxic of the three and ortho-cresol the least toxic. The behaviour of the fish conformed well to the descriptions given by other workers. In the stronger solutions the sense of balance is lost almost at once, the fish falling over on to its side; there are wild, dashing movements at first, later only feeble attempts at swimming and shallow, flickering opercular movement. In the weaker solutions the sense of balance is also affected very quickly, the fish falls over but may regain its equilibrium temporarily a number of times; finally, however, it loses all power of co-ordinated movement and remains on its side with occasional attempts at swimming and respiratory movements which are almost imperceptible. This helpless condition prevails for the rest of the survival time, the death point being rather ill-defined.

Twelve reaction experiments were carried out with phenol at concentrations of 0·04–0·0003%, and four representative results are given in Fig. 2. The minnows showed little or no capacity for recognizing and avoiding the solution at any concentration. In the 0·04% experiment the phenol was admitted on the right when

the fish was in the left half of the tube; however, it entered the solution at once, there it gulped and moved very jerkily, visited the water zone and returned twice, at 2 min. lost its sense of balance, swam wildly up and down the tube about six times, and finally rested on its side almost motionless. In the 0·01 % experiment the phenol

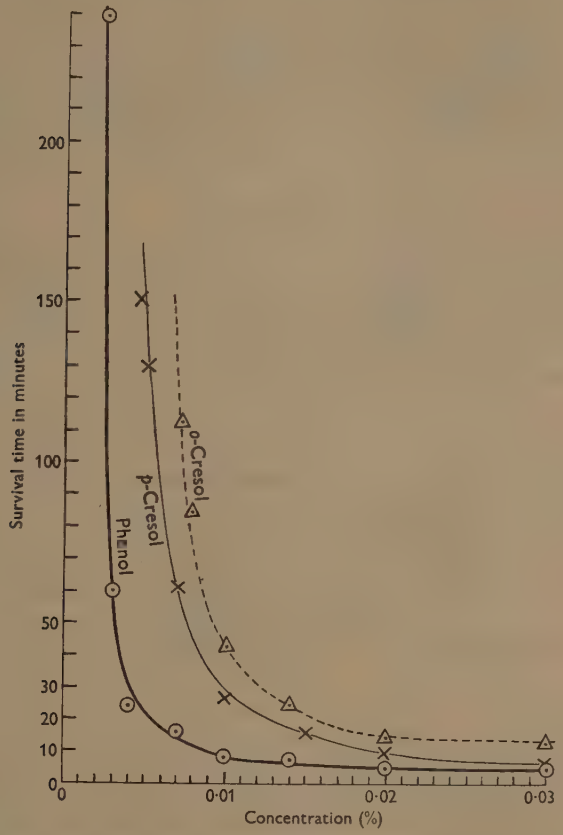


Fig. 1. Survival times for minnows in solutions of phenol, ortho-cresol and para-cresol. The points are means of three determinations. Temp. 17° C. pH of solutions 6·6–6·8.

was run in on the side occupied by the minnow; for over a minute the fish displayed no sign of irritation, floating gently on the current towards the water zone. At 4 min. symptoms of intoxication suddenly developed with wild swimming up and down, and at 6 min. the sense of equilibrium was quite lost; the fish now found its way into the water where it kept up feeble movement; at 8 min. it had recovered to a considerable extent and was swimming upright once more. At 9–10 min. the water-phenol flow was reversed, but again the minnow showed no power of discriminating between

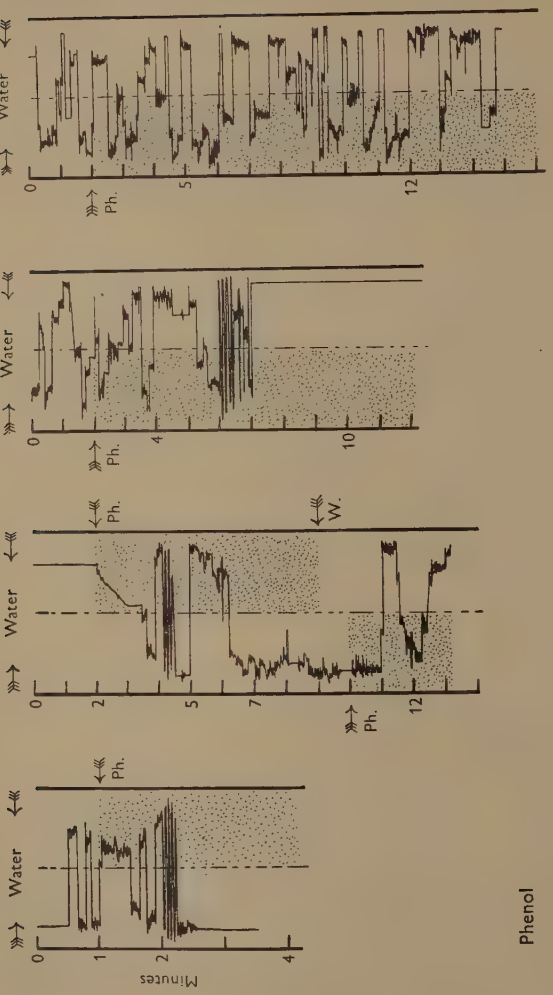


Fig. 2. The reactions of minnows to 0·04, 0·01, 0·004 and 0·0004 % solutions of phenol. pH of water 6·8, of solutions 6·6–6·8. Temp. 16·5–17·5° C. Survival times at these concentrations (fish continuously immersed in the solution) 4 min., 8 min., 24 min., 40–50 hr.

water and solution and at 13 min. lost its sense of balance for the second time. It recovered quickly on being placed in an aquarium.

The same result is evident at 0·004%; the fish visited the solution several times, and this resulted in intoxication and collapse. At 0·002% minnows would enter the solution half of the tube, remain almost motionless for 1–2 min. and then fall over. A 0·001% solution is much less rapid in its effect, and a spell of several minutes in the phenol does not produce anything worse than a slight uneasiness and irregularity in respiratory movement. The 0·0004% experiment included in Fig. 2 was run for 13 min., during which time the fish crossed the water-solution junction at least 30 times, but no avoiding action or symptoms of intoxication developed. This concentration is near the threshold of toxicity for phenol.

IV. PARA-CRESOL, $CH_3C_6H_4OH$

Twelve experiments were run with para-cresol covering the concentration range 0·03–0·002%. Four records are given in Fig. 3; this includes two results for 0·03% to illustrate the good measure of agreement observed in different experiments. At 0·03%, in marked contrast to what is seen in the case of phenol, a most definite avoiding action was evident. The minnows would not enter the solution at all; if they swam to the water-solution junction they would halt, make some gobbling respiratory movements and retreat. Para-cresol is so toxic at this concentration that even these momentary contacts were sufficient to produce some measure of intoxication, and the fish appeared somewhat distressed at the end of the experiment but had not lost their sense of balance. At somewhat greater dilutions the animal's capability of detecting and avoiding the solution seems to diminish very sharply. A fair measure of avoiding action was evident at 0·02%, but at 0·01% (Fig. 3) the fish penetrated the cresol about six times, and this resulted in a sudden attack of furious swimming up and down ending in collapse. Recovery began at 8 min. At still greater dilution the water-solution junction is usually crossed with no hesitation; in experiments at a concentration of 0·004 and 0·003% the fish appeared to be less happy in the solution than in the water, and an extremely vague negative reaction might be observed. In the fourth record in Fig. 3 this vague tendency to prefer the water zone was shown at the beginning of the experiment; at 12 min., however, the minnow swam far into the solution and remained motionless for nearly half a minute. At 0·003% the intoxicating power of para-cresol solutions is declining, and at the end of the experiment the fish was swimming more or less normally.

The writer has shown (1948) that the minnow will display a definite avoiding reaction to very dilute lead nitrate solutions. This avoiding reaction is quickly developed; at 10^{-3} N it seems immediate and at 10^{-5} N appears in about 5 min. These solutions take a considerable time to produce any marked toxic effect; the survival time of minnows in 10^{-3} N-lead nitrate is about 4 hr., and during the greater part of this time the fish retains its sense of balance and swims more or less normally. *Gasterosteus aculeatus*, similarly, will establish an avoiding reaction to 10^{-4} N-lead nitrate in about 5 min.; at this concentration the survival time is about 10 hr., and the only toxic symptom which develops in the first hour is a gradual rise in the rate

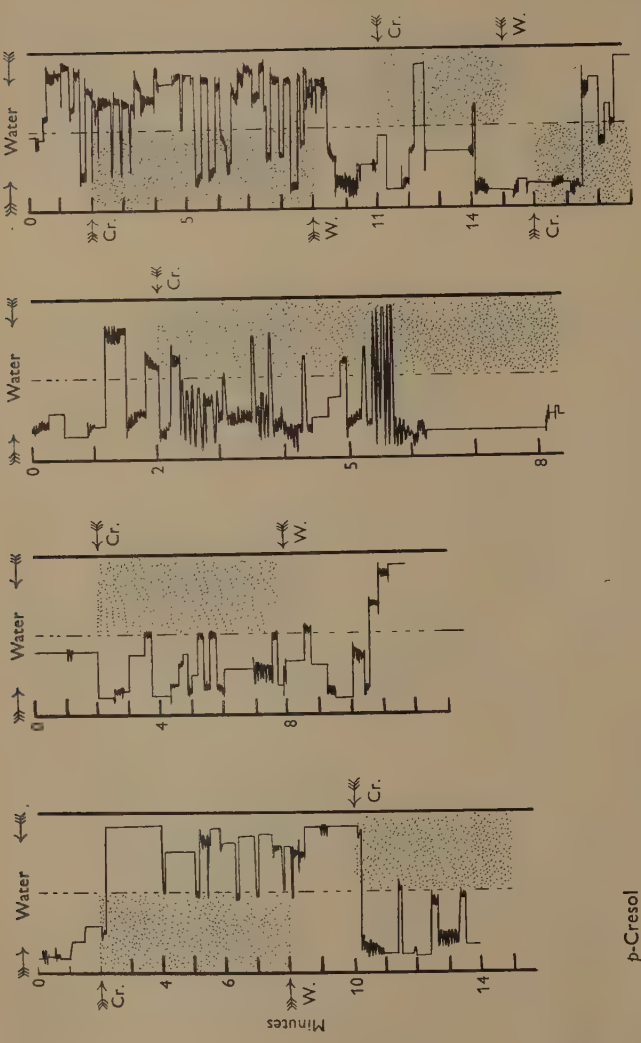


Fig. 3. The reactions of minnows to 0·03 (two records), 0·01 and 0·003 % solutions of para-cresol. pH of water and solutions 6·8. Temp. 17·5–18° C. Survival times at these concentrations (fish continuously immersed in the solution) 7 min., 27 min., 4–5 hr.

of the respiratory movements. The general position in the case of zinc salts is similar. In an experiment with *Pygosteus pungitius* (Jones, 1947, p. 117) it was found that after 36 min. immersion in a 0·01 N-zinc sulphate solution (fatal in about 100 min.) the fish would quickly select the water zone of the apparatus. Lead and zinc salts kill

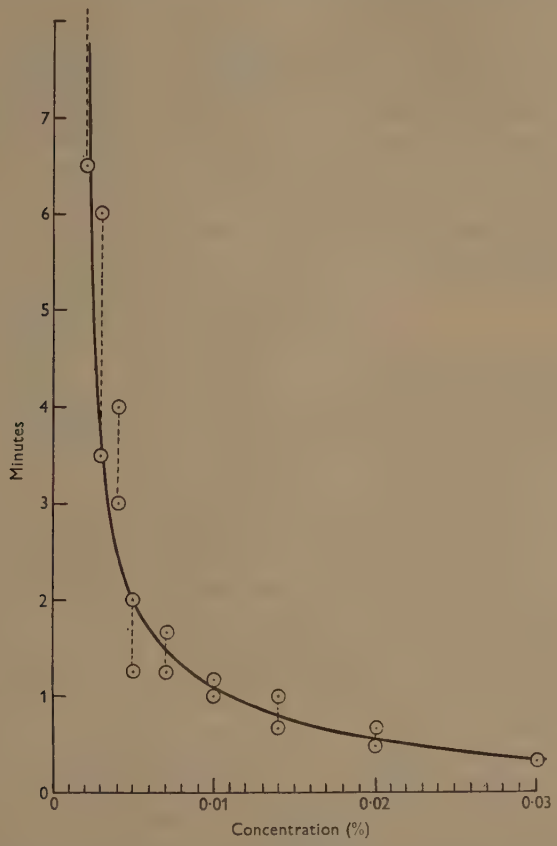


Fig. 4. The effect of para-cresol solutions on the minnow's sense of balance; the lower points are the times at which the fish begin to have difficulty in swimming upright, and the upper points are the times at which the fish appear to lose completely their sense of balance and ability to swim in a co-ordinated manner. All times are very approximate. Temp. 18° C.

fish by impairing the respiratory efficiency of the gills and appear to have no specific effect on the nervous system; the time taken by the animal to establish an avoiding reaction to the toxic solution is a small fraction of the time this takes to destroy its capability of doing so. The general situation in the case of para-cresol is very different; here it would appear that the solution has two distinct effects, an irritating effect which provokes the avoiding reaction and an intoxicating effect which destroy's the animal's

power of swimming in a co-ordinated manner. The irritating effect is probably predominant at high concentrations, so that an avoiding reaction is immediately or very quickly established, but at lower concentrations the solution rapidly becomes less effective as an irritant but little less effective in intoxicating power; the time the fish takes to avoid the solution and the time this takes to destroy its capability of doing so thus come to be separated by the narrowest of margins. At 0·03% para-cresol the sense of balance begins to fail in about 20 sec. (see the data set out in Fig. 4), but as the fish will avoid the para-cresol zone almost immediately it escapes intoxication. At 0·01% para-cresol the fish does not appear to be irritated by the solution but persists in venturing into it; the paralysing effect at this concentration is little less rapid than at 0·03%, and so an avoiding reaction is never established. At 0·003% it is probable that the solution has a sufficient irritating effect to induce some measure of preference for the water zone, but at this dilution only about 4 min. immersion in the solution is needed to disturb the animal's equilibrium and the situation is still critical.

V. ORTHO-CRESOL, $CH_3C_6H_4OH$

With ortho-cresol eight experiments were run over the concentration range 0·04–0·003%, and four are recorded in Fig. 5. The results are very similar to those obtained with para-cresol; at 0·04% the fish avoided the solution very well, but at greater dilutions the animal's capability for recognizing the solution seems to decline rapidly, for at 0·02% the fish persisted in entering it with the usual result. In the 0·01% experiment the minnow became intoxicated very quickly, and after the usual series of wild rushes up and down stopped on its side in the water zone. Here it regained its equilibrium in about 3 min., at 10 min. swam into the solution, had a second fit of intoxication, settled in the water and at 16 min. was recovering again. At 0·003% the same inability to recognize the cresol was evident, but the symptoms of intoxication took several minutes to develop.

In addition to the foregoing experiments with pure solutions of phenol and cresol a number of trials were made with various dilutions of a phenolic effluent supplied by a firm of manufacturing chemists. The neat effluent was a clear solution of pH 6·8 and strong 'carbolic' odour. The total concentration of phenolic substances, including phenol, cresols and a very small amount of xylenol, was stated to be approximately 0·0762% w/v; the chief other substances present were sodium sulphite, sodium chloride and sodium sulphate. The behaviour of the minnows was essentially similar to that observed in the experiments with pure solutions of cresols. To a 1 : 3 dilution they reacted very definitely, refusing to enter the solution at all and displaying obvious signs of great irritation on encountering it; continuously immersed in the solution they lost their sense of balance almost at once and died in about 10 min. A 1 : 6 dilution also produced an avoiding reaction but of rather less definite form. A 1 : 10 dilution of the effluent, fatal in 35 min., did not appear to be recognized, the fish entering it with no hesitation, and 1 : 20, 1 : 40 and 1 : 100 dilutions similarly produced no response.

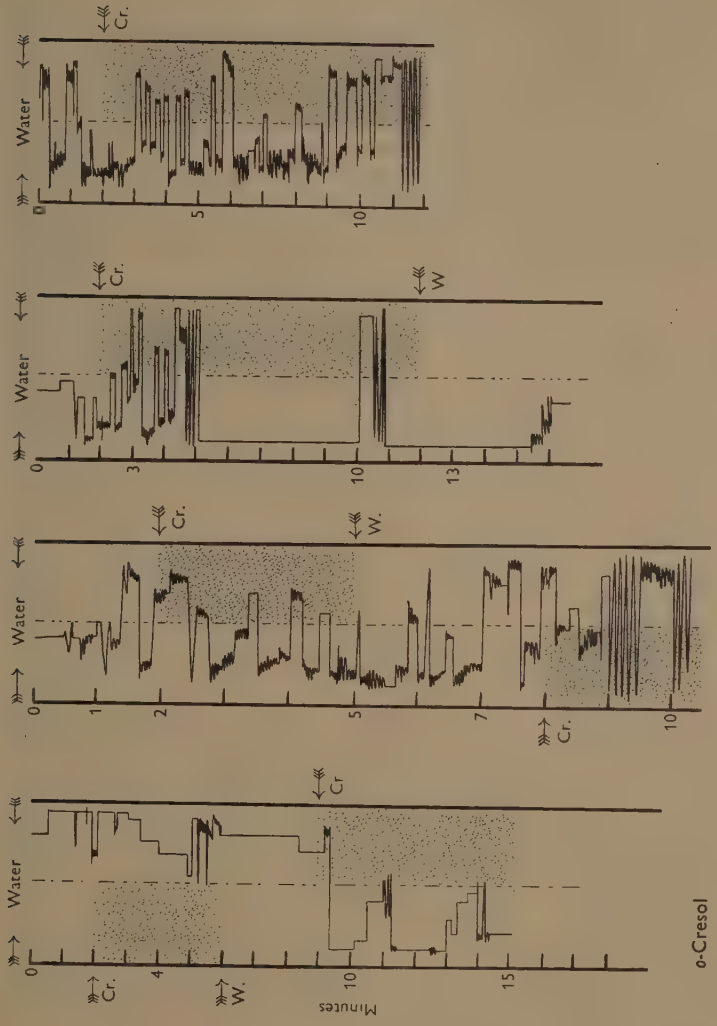


Fig. 5. The reactions of minnows to 0·04, 0·02, 0·01 and 0·003 % ortho-cresol. pH of water and solutions 6·8. Temp. 17–18° C. Survival times at these concentrations (fish continuously immersed in the solution) 13 min., 15 min., 43 min., 22 hr.

VI. GENERAL OBSERVATIONS

In the case of phenol there appears to be little chance of fish avoiding a solution of any concentration, and while high concentrations of ortho- and para-cresol may be detected and avoided the animal does not immediately reject solutions of greater dilution whose power of upsetting its sense of balance is little inferior. Once the power of co-ordinated movement is lost there can be little possibility of developing any definite negative reaction. Phenol and the cresols are therefore highly dangerous polluting substances, but their toxicity falls off very quickly with decrease in concentration, and it is probable that at great dilution they are not as stable as lead, copper and zinc salts. Hence it is possible that a slight degree of pollution may do no harm, provided that the effluent is immediately diluted to the maximum degree possible and not allowed to run into pools where it may mix very slowly with the stream water to form solutions of comparatively high concentration into which the fish may venture.

SUMMARY

This paper records some observations on the reactions of minnows to phenolic substances. The fish are placed in a horizontal tube, half of which is filled with flowing water and half with flowing solution of the concentration to be tested. Water and solution are very sharply differentiated.

Solutions of phenol, ortho- and para-cresol are highly toxic and appear to have some specific effect on the nervous system of fish, causing them to lose their sense of balance and capability of co-ordinated movement. The time the solutions take to produce this effect is much shorter than the survival time; in a 0·01% solution of para-cresol, for example, a minnow loses its sense of balance in about 70 sec. but takes about 30 min. to die.

Over the concentration range 0·04–0·0004% minnows appear to have little or no power of discriminating between phenol and water, swim into the solution and become intoxicated.

At concentrations of 0·03–0·04% minnows will avoid para-cresol and ortho-cresol solutions immediately and escape intoxication. At somewhat lower concentrations the fish venture into the solution, and this quickly destroys their capability of recognizing and avoiding it. The situation is markedly different from that observed in the case of lead and zinc salts; here the time the fish takes to establish an avoiding reaction to the solution is very much shorter than the time this takes to destroy its capability of doing so.

REFERENCES

ALEXANDER, W. B., SOUTHGATE, B. A. & BASSINDALE, R. (1935). Survey of the River Tees. Part 2. *Tech. Pap. Wat. Pollut. Res., Lond.*, no. 5.

EDMUNDS, C. W. & GUNN, J. A. (1936). In A. R. Cushny's *Text-book of Pharmacology and Therapeutics*, 11th ed. London.

ELLIS, M. M. (1937). *Bull. U.S. Bur. Fish.* **48**, no. 22.

JONES, J. R. E. (1947). *J. Exp. Biol.* **24**, 110.

JONES, J. R. E. (1948). *J. Exp. Biol.* **25**, 22.

MASON-JONES (1930). *Detailed Biological and Chemical Reports on Tars used for Road-Surfacing.* Ministry of Transport and Ministry of Agriculture and Fisheries. London: H.M. Stationery Office.

SHELFORD, V. E. (1917). *Bull. Ill. Nat. Hist. Surv.* **11**, 381.

THE BODY TEMPERATURE OF WOODLICE

By E. B. EDNEY

From the Zoology Department, University of Birmingham

(*Received* 17 *November* 1950)

(With Four Text-figures)

INTRODUCTION

The present work forms part of an investigation into the water relations of woodlice. It has already been shown (Edney, 1951) that, during short exposures, woodlice can tolerate a higher temperature if the air is dry than if it is saturated with water vapour, and the experiments to be described were designed to estimate more precisely the effect of evaporation of water on the body temperature of the animals concerned. Measurements of the body temperature of insects under various environmental conditions have been made by several workers and reviewed by Gunn (1942), but no comparable data for woodlice are available, and since they apparently lack the efficient waterproofing layer in the integument which is characteristic of the majority of terrestrial insects, they might be expected to show greater temperature effects due to evaporation.

METHODS

In measuring the temperature of small animals such as woodlice, the instruments themselves may introduce considerable error. Where thermocouples are used, as in the present work, the two main sources of error are the risk of damage to the animal and conduction of heat by the instrument, and for these reasons the usual thermo-needles are unsatisfactory. It is not possible entirely to avoid error, but the amount can be greatly reduced if the instrument used is small and smooth, and this was aimed at in the construction of the thermocouples used in the present experiments. The junctions were made of 48 s.w.g. copper and constantan wire and coated with bakelite varnish. The wires were silver-soldered together end to end. This can be done, using a binocular microscope and a very small gas jet, so that there is no appreciable increase in the diameter of the wire. The joined wire is then coated with varnish and baked, after which the wires are bent at the joint so that they lie parallel. A short piece of narrow-bore glass tubing slipped over the two wires and varnished into position leaving rather less than 1 cm. projecting with the junction itself at the tip, provides a useful hold for the forceps when inserting the thermocouple and a protection against damage to the insulating varnish.

As regards the accuracy of this instrument, Shakespeare (*in litt.*) has shown empirically that where a thermojunction composed of 46 s.w.g. copper and constantan is immersed in a body whose temperature is 35° C. higher than that of the air through which the junction wires run, conduction of heat away from the body by the wires will not lead to an error greater than 0·01° C. provided the junction is at least 2·0 mm.

inside the body. (Empirical data for large thermo-needles were determined by Gunn *et al.* (1948).) In the present experiments, conditions were well within these limits, so that errors in temperature as a result of conduction by the thermocouple wires can safely be neglected.

The apparatus used is shown diagrammatically in Fig. 1. The experimental chamber itself is a glass vessel, 2·5 in. in diameter and 4 in. long, through which a stream of air can be passed. At the outlet end a large rubber bung carries a glass tube through which air is extracted by a small pump (*P*). The air is then dried and brought to the required temperature by passing through a copper coil which is immersed in a tank of water together with the experimental chamber, the temperature of the water being controlled to within 0·1° C. The air then re-enters the experimental chamber. Inside the chamber a perspex framework attached to the bung

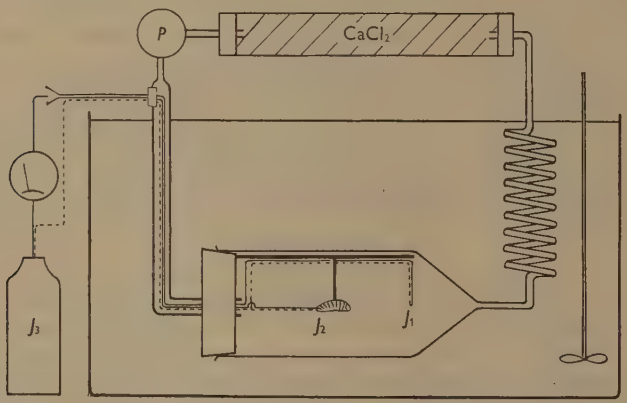


Fig. 1. Apparatus used for measuring the temperature of woodlice under controlled conditions. $\mathcal{J}_1$ and $\mathcal{J}_2$, thermojunctions in the oncoming air and the animal respectively; $\mathcal{J}_3$, constant-temperature junction; *P*, air pump.

carries terminals for the fine thermocouple wires and an arm for holding the animal during an experiment. There are two thermojunctions inside the experimental chamber, the first ($\mathcal{J}_1$) is supported in front of the animal in the advancing air stream, the second ($\mathcal{J}_2$) is inserted into the rectum of the animal, the latter being held by a loop of thread which emerges from the hollow supporting arm and which can be adjusted from outside the chamber. (This thread is not shown in the diagram.) From the terminals mentioned above, thicker insulated wires (two copper and one constantan) run through the glass tube to above water-level, where they emerge through an air-tight joint and run to the 'cold junction' ($\mathcal{J}_3$) and galvanometer. The cold junction used was a large thermos flask of water at 17° C. There was no noticeable change in the temperature of this junction as measured by a mercury thermometer in as long as 12 hr., and since an experiment usually lasted for about 1 hr., the constancy of the cold-junction temperature can be assumed. By means of a simple

switch either junction J_1 or J_2 can be brought into circuit. Air was pumped round the circuit at 1 l./min.

In experiments such as these, the value of the results is of course limited by the accuracy of the measuring instruments. Many thermojunctions were made; each was calibrated separately, and no junction was used unless during calibration it was free from hysteresis for either rapidly or slowly changing temperatures and gave repeatable readings within 0·1° C. During calibration the junction was immersed in stirred water whose temperature was read by a mercury-in-glass thermometer graduated to 0·1° C.

Before experiment, the animals were all kept at room temperature in a saturated atmosphere. The animal to be used for experiment was lightly anaesthetized with ether to facilitate handling; the thermocouple junction was then inserted into the rectum through the anus for a distance of about 4 mm. If any sign of damage to the animal was visible as a result of this insertion, it was discarded and another animal used. (With practice it was usually possible to insert the junction easily and without apparent damage.) The animal was then attached to the holder, either by a drop of wax or by the fine thread described above (the procedure used made no difference to the results); the rubber bung with its attached frame carrying thermojunctions and animal was then inserted into the glass chamber and the whole immersed in the tank of water. Air of the required temperature and humidity was then allowed to flow past the animal (rate of flow 1 l./min.), and the temperatures of the oncoming air and of the animal were read every 2 min. or every 5 min. according to the nature of the experiment.

If the animals were alive when exposure commenced they would usually remain alive during a 45 min. exposure, except in dry air at 37° C. There was, however, no difference in amount of cooling between living and dead animals, so that this factor can safely be neglected.

EXPERIMENTAL RESULTS

(*a*) *The effect of warm dry air on the temperature of* Ligia

The first experiments were carried out on *Ligia*, the largest of the British terrestrial isopods. Dry air at 30° C. was used in these experiments. After the temperature of the animal had reached its lowest point, the dry air was replaced by saturated air which had been bubbled through a series of vessels containing water at the same temperature as the air inside the chamber. After the temperature of the animal had again settled down (11 min.), dry air was once more introduced to replace the moist.

Fig. 2 is a composite curve derived from several such experiments—the initial period of equilibration is not shown. During the first 'dry' period, the lowest body temperature of the animal was 6·2° C. (mean of four determinations, varying from 5·9 to 6·4° C.) below that of the surrounding air at 30° C. As soon as saturated air was introduced, however, the temperature of the animal rose rapidly until it reached the same temperature as the surrounding air. During the second 'dry' period the temperature again fell, rather more slowly than it had risen, and not quite so far as in the first dry period.

This experiment was repeated using a dead animal with results which were so similar to those from the living animal as to make a second curve unnecessary.

(*b*) *A comparison with other species of woodlice*

Data derived from the type of experiment described above are satisfactory in providing a general picture of what is going on in the particular animal used, but the information is too qualitative for comparisons to be made between one species and another. Data derived from experiments in which the temperature of the air rises (several such experiments were done) are also unsatisfactory in this respect, since it is not easy to control the rate of rise of temperature, and there is no means of telling

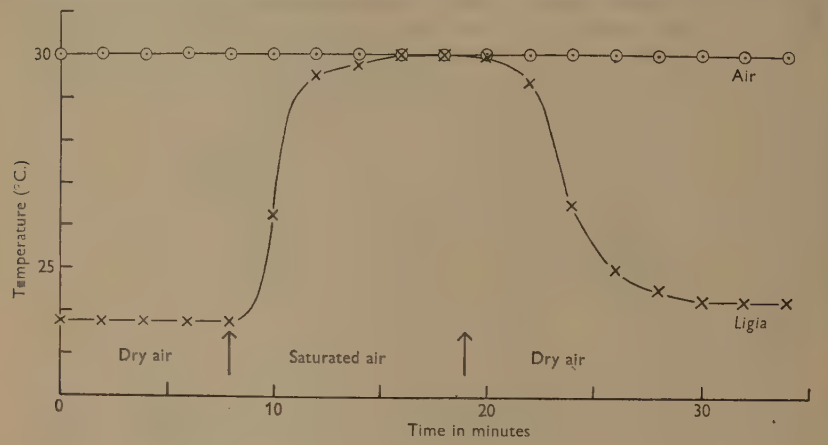


Fig. 2. Temperature curve for living or dead *Ligia* exposed to slowly moving air at 30° C.

to what extent, owing to its thermal capacity, the temperature of the animal lags behind that of the air.* The best one can do towards making quantitative comparisons between species is to use a constant temperature and humidity, and to choose an arbitrary time after the commencement of the experiment (when each species is giving fairly constant readings), at which to measure the cooling effect.

Experiments with this end in view were carried out using *Armadillidium vulgare*, *Oniscus asellus*, *Porcellio scaber*, *Ligia oceanica*, and the insect *Blatta orientalis*. In one series of experiments a constant air temperature of 37° C. was used, in another, 20° C., and the air was dry. The experiment was repeated with at least three individuals of each species as alike in size as possible. In another series individuals of each species were subjected to the same treatment except that the air was saturated with water vapour. In all these experiments readings were taken every 5 min. and continued until the temperature had settled down for at least 10 min. This meant an exposure of about 40 min. for all species except *Ligia* whose temperature did not settle down even after 2 hr. exposures.

* See discussion on p. 278.

The results are shown in Table 1, and a selection of them are graphed in Figs. 3 and 4. The curve shown for any one species is the curve for that specimen which, by reason of its central position, is most representative of the group. An indication of the variation encountered is shown, where relevant, by vertical lines at the end of each curve; this represents the variation at that time and does not *necessarily* correspond in all cases with the variation shown in Table 1 which is based on readings taken 30 min. after the commencement of each experiment.

Table 1. *Depression of body temperature below that of a surrounding stream of air at either* 20 *or* 37° C., *after* 30 *min. exposure*

	Air temperature 20° C.				Air temperature 37° C.			
	No. of observations	Depression in ° C.			No. of observations	Depression in ° C.		
		Mean	Max.	Min		Mean	Max.	Min.
Ligia	3	2·6	3·0	2·3	3	6·8	7·3	6·8
Oniscus	3	1·5	1·6	1·3	3	2·7	3·0	2·5
Armadillidium	3	0·5	0·5	0·4	4	1·8	2·0	1·5
Porcellio	3	0·4	0·4	0·3	4	1·3	1·5	1·1
Blatta	3	0·7	0·7	0·6	3	2·4	2·4	2·3

The position on the ordinate from which each curve runs is, of course, quite without significance for the present purpose; it depends merely upon the temperature of the animal when the first reading was taken, which may have been anything up to 2 min. after assembly of the animal in the apparatus.

It is clear from these curves that the arbitrary time of 30 min. after the beginning of an experiment chosen for the comparative figures given in Table 1 is not entirely satisfactory; the temperature of *Ligia*, for example, continues to rise slowly throughout the experimental period, and that of *Blatta* at 37° C. is not quite steady. However, all the other species used give constant readings from about 25 min. onwards, so that the comparison is worth making.

In all experiments where the air was saturated, the temperature of the animals was, within the limits of accuracy of the apparatus, the same as that of the air, so that the depressions of temperature in dry air are certainly due to evaporation. Since they are all the same, the curves for saturated air are not shown in the figures.

When the depressions shown by each species are compared, it is apparent that the amount varies considerably from one to another, and that the depression is greater when the air temperature is 37° than when it is 20° C. *Ligia* shows by far the greatest depression, as much as 7 from 37° C.; next comes *Oniscus*, while *Armadillidium* and *Porcellio* show the least depression. The order of the species is the same at both temperatures, and corresponds with the order as regards rate of evaporation which was found previously (Edney, 1951), with the exception of the relative positions of *Armadillidium* and *Porcellio* at 37° C.; but at that temperature, both the evaporation rates and temperature depressions of the two species are close together, so that little significance can be attached to their relative positions. These results are discussed further below.

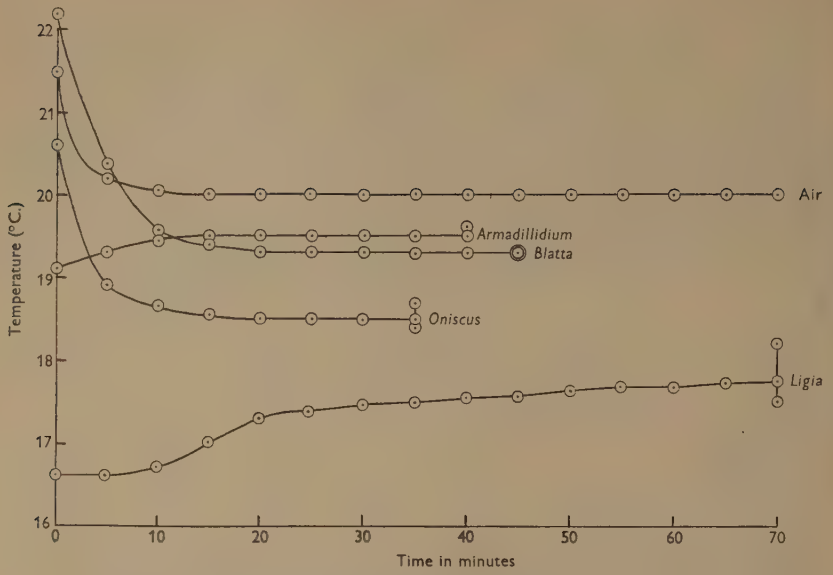


Fig. 3. Temperature curves for various woodlice and the cockroach, *Blatta*, exposed to slowly moving dry air at 20° C.

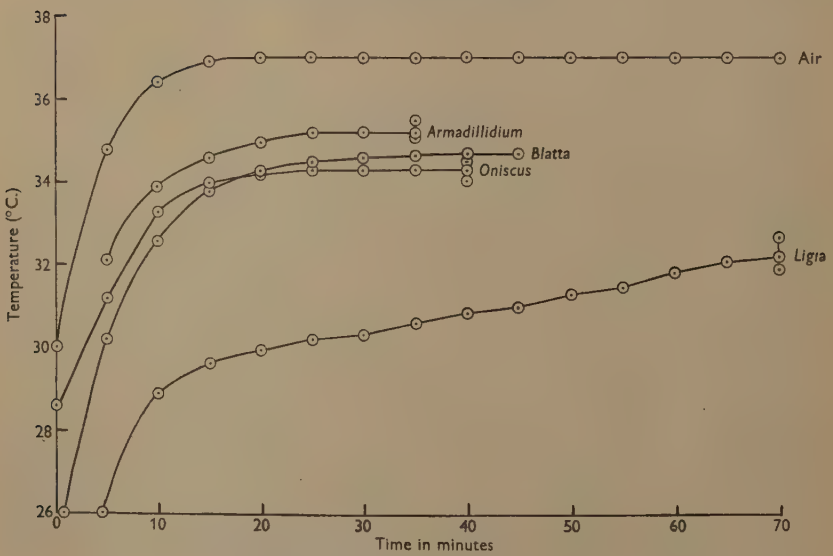


Fig. 4. Temperature curves for various woodlice and the cockroach, *Blatta*, exposed to slowly moving dry air at 37° C.

It is interesting to notice that the insect *Blatta*, which is approximately the same size and shape as *Ligia*, shows a very much smaller temperature drop than the latter, an observation which corresponds with what is known of the permeability of the insect cuticle as compared with that of woodlice.

Direct estimation of the water lost during some of these experiments was made by weighing the animals before and after exposure. At 20° C. a specimen of *Ligia* which showed a temperature depression of 2·6° C. lost 0·06 g. water/hr., while an *Armadillidium* which showed a temperature drop of 0·4° C. lost 0·002 g. water/hr. *Ligia*, therefore, lost thirty times as much water as *Armadillidium*, and its surface area was only five times greater, so that the rate of evaporation per unit area was six times greater from *Ligia* than from *Armadillidium*, and a more pronounced drop in temperature was to be expected. Another such comparison was made between *Ligia* and *Blatta* at 37° C. The dimensions of the two animals were approximately the same, yet *Ligia* lost two and a half times as much water as *Blatta*, and its temperature depression was correspondingly greater (6·8° C. as compared with 2·4° C.).

DISCUSSION

The experiments described have shown a depression in the body temperature of woodlice below that of the environment. Results of this kind may be considered from two points of view: first, they provide biophysical information relating to permeability of the integument and allied phenomena, and secondly, they provide ecological information concerning the distribution and evolution of woodlice. Considered biophysically it is important to know just what physical or physiological factors produce the temperature depressions recorded; considered ecologically, the precise interplay of causes is not so important as the aggregate effect. The present results will be considered from the biophysical aspect first.

When, during the course of an experiment, a steady temperature is reached, total heat lost by the animal must be equal to total heat gained. Heat is gained from the environment by radiation and conduction, and from within by metabolic heat. Under the conditions used, heat gained by metabolism is unimportant, for if metabolic heat contributed significantly to the balance one would expect to find the temperature of the animal above that of the environment when loss of heat by evaporation was prevented in saturated air. In fact such a rise in temperature was never greater than 0·1° C.

Gain of heat by radiation is an unknown factor, but in all probability it is low. By far the greatest gain of heat is by conduction from the air, conduction by the wires of the thermocouple having been shown above probably to be negligible. Similarly, the only important means by which heat is lost (radiation again being doubtful but probably very low) is evaporation of water from the surface.

It is approximately true to say, therefore, that the final equilibrium temperature reached is that at which heat gained from the air just balances heat lost by evaporation; and since both processes are roughly proportional to surface area, then provided the rates *per unit area* remain constant, the equilibrium point will be independent of size. It can be assumed that, under the conditions used, the rate of gain of heat from

the air is not significantly affected by differences in cuticle structure between one species and another, so that the observed specific differences in equilibrium temperature can be wholly ascribed to differences in rates of evaporation, and species which show greater rates of evaporation may be expected to show greater temperature depressions (although the two variables may not be strictly proportional) no matter what their size.

On the other hand, the speed of equilibration will be slower in larger animals, for the volume (and hence the thermal capacity) of a body of constant shape increases more rapidly with length than does surface area. For this reason, the relative speed of equilibration in various species bears no relation to the permeability of their integuments, and information about the latter can only be obtained by a comparison of equilibrium temperatures.

There are no previous data for body temperatures of woodlice, but the present results may be compared with those obtained for various insects. Several authors, e.g. Necheles (1924), Mellanby (1932) and Koidsumi (1935), have measured the effect of dry air on the body temperature of large insects, and in general they have found temperature depressions of from 3 to 5° C. when air temperatures are high (about 40° C.). Knowing as we do that the permeability of the insect integument is usually a good deal lower than that of woodlice, these figures may appear rather high; but as Ramsay (1935), Wigglesworth (1945) and others have shown, the insect cuticle undergoes a change at temperatures which vary from one species to another (about 35° C. for *Blatella*) leading to much greater permeability, so that large depressions at high temperatures are not unexpected. Even so, the depressions found are not so great as that shown by *Ligia* at 37° C.

Not many measurements of small insects' temperatures have been made—surely the most ambitious attempt is that of Vinogradskaya (1942) who measured the temperature in different parts of the mosquito *Anopheles maculipennis*. She claims to have found a body temperature up to 1° C. higher than the surrounding air between 5 and 25° C., with saturation deficiencies up to 10 mm. Hg. Above 25° C. she finds a body-temperature depression up to 3° C. in dry air. Such temperatures are not, of course, impossible, but they must be extremely difficult to measure accurately.

Another respect in which woodlice differ from some insects is the behaviour as regards temperature depression of dead as compared with living animals. In the present work, no difference at all was found, whereas in insects the temperature depression is often greater in living than in dead individuals. Thus Buxton (1924) found that the temperature of a living *Adesmia* beetle exposed to direct sunlight on sand was only 0·5° C. above the sand temperature, but rose to about 6° C. above if the insect was dead. (Koidsumi (1935), however, found no such difference in the insects with which he worked.) The explanation of this anomaly may lie in the difference between the site of water loss in insects and woodlice; in the former, the greater part of evaporation takes place through the spiracles, and if these are closed after death, evaporation is reduced and the temperature depression is less. In woodlice, on the other hand, the absolute amount of water lost through the respiratory surfaces is not more than a fifth of the total amount (Edney, 1951), evaporation takes

place from the whole body surface, and this undergoes no significant change at death.

Turning now to the ecological implications of the present results, we may ask whether the temperature depressions shown, no matter how they are caused, are of survival value, and if so, whether they are of significance in the evolution of the group.

The impression gained from studying the effect of humidity upon upper tolerable temperature limits, that the ability to evaporate water rapidly will enable woodlice to survive short exposures to temperatures which would otherwise be fatal, is confirmed by the present results. *Ligia* for example is killed by a 15 min. exposure to 34·5° C. in saturated air, but it can withstand 41·5° C. in dry air, and this difference corresponds well with the temperature depression of 7 from 37° C. which has now been measured. The effect is smaller in other genera, but even in *Armadillidium* a depression of 1·8° from 37° C. occurs, and may well be of survival value. If we suppose woodlice to have evolved from maricolous to terricolous forms via the littoral zone, then the capacity to keep cool by rapid evaporation during short periods of exposure to high temperatures would have been of great value, as it may well prove to be in the littoral form *Ligia* to-day. Unfortunately, we do not yet know anything of the precise microclimatic conditions to which woodlice are subjected in the field, so that the picture remains for the present incomplete.*

On the other hand, as compared with insects, woodlice have little real advantage, for although insects usually are not able to cool themselves so much as woodlice by rapid evaporation, they have developed a higher temperature tolerance, and this, so far as adaptation to terrestrial conditions is concerned, is of much greater value.

SUMMARY

1. Measurements by means of thermocouples, accurate to 0·1° C., were made of the body temperature of the woodlice *Armadillidium*, *Porcellio*, *Oniscus* and *Ligia*, and of the cockroach *Blatta*, both alive and dead, in a stream of saturated or dry air at both 20 and 37° C.

2. No difference in temperature depression was found between living and dead woodlice, and in all the animals used there was, after equilibration, no difference greater than 0·1° C. between the air temperature and body temperature if the air were saturated with water vapour.

3. In dry air, the body temperature of all the animals except *Ligia* settled down after at most 25 min. to a steady temperature which was lower than that of the surrounding air. The body temperature of *Ligia* continued to rise slowly for at least 2 hr., though remaining well below that of the environment.

4. After 30 min. in dry air at 20 and 37° C. respectively, mean temperature depressions (of at least three readings at each temperature for each species) were, in degrees centigrade: *Ligia*, 2·6 and 6·8; *Oniscus*, 1·5 and 2·7; *Porcellio*, 0·4 and 1·3; *Armadillidium*, 0·5 and 1·8; and the cockroach *Blatta*, 0·7 and 2·4. The order of the

* Parallel evidence bearing upon differential adaptation to terrestrial conditions has recently been demonstrated in the allied field of excretion by Dresel & Moyle (1950).

species in this respect is substantially the same as their order in respect of evaporation rate, which was established previously.

5. Certain anomalies which appear when these figures are compared with previously established figures for insects are probably the result of differences in permeability of the integument and in the site of water loss. The ability to evaporate water rapidly, and thus to cool the body, may be of survival value when woodlice are exposed to high temperatures for short periods, particularly in littoral forms which may well have been intermediate in the evolution of terricolous from maricolous isopods.

The above work was carried out while in receipt of a grant from the Agricultural Research Council. My thanks are due to Dr J. A. Ramsay for his most helpful observations upon the manuscript of this paper. I am also indebted to Dr G. A. Shakespeare for permission to quote unpublished work, and to Mr R. Priest for his painstaking assistance.

REFERENCES

BUXTON, P. A. (1924). *Proc. Roy. Soc.* B, **96**, 123–31.
DRESEL, E. I. B. & MOYLE, V. (1950). *J. Exp. Biol.* **27**, 210–25.
EDNEY, E. B. (1951). *J. Exp. Biol.* **28**, 91–115.
GUNN, D. L. (1942). *Biol. Rev.* **17**, 293–314.
GUNN, D. L. *et al.* (1948). *Anti-Locust Bull.* no. 3.
KOIDSUMI, K. (1935). *Mem. Fac. Sci. Tokyo*, **12**, 281–380.
MELLANBY, K. (1932). *J. Exp. Biol.* **9**, 222–31.
NECHELES, H. (1924). *Arch. ges. Physiol.* **204**, 72–86.
RAMSAY, J. A. (1935). *J. Exp. Biol.* **12**, 373–83.
VINOGRADSKAYA, O. N. (1942). *Zool. Zh. Moscow*, **21**, 187–95.
WIGGLESWORTH, V. B. (1945). *J. Exp. Biol.* **21**, 97–114.

OVIPOSITION BEHAVIOUR IN THE TWO STRAINS OF THE RICE WEEVIL, *CALANDRA ORYZAE* LINN. (COLEOPT., CURCULIONIDAE)

By F. SEGROVE

The Department of Zoology, The University of Sheffield

(*Received 7 December* 1950)

(With Six Text-figures)

A knowledge of the biotic potential of an insect population, i.e. of its rate of multiplication under various conditions, is of obvious importance in the case of grain-infesting insects. Such knowledge is available for very few species, for its computation requires exact information of certain biotic constants (Chapman & Baird, 1934) which can be determined only by protracted experiment. Not the least important of these is one which is a function of the fertility and rate of oviposition, and the primary aim of the present investigation has been to obtain information on this subject for the rice weevil, *Calandra oryzae*.

Previous work on oviposition has frequently fallen short of requirement for several reasons. Where full-scale experiments have been carried out (e.g. Kunike (1936) and Lavrekhin (1937)), the control of environmental conditions has been too inadequate for the results to have any great value. Where a more rigid control has been attempted short-term experiments on rates of oviposition have usually been conducted (e.g. MacLagen & Dunn (1936), Crombie (1942), Richards and his co-workers (1944, 1947)), and not infrequently selected females have been employed. The results, in consequence, though of value in other directions, are seldom if ever a reliable index of total fertility and throw little light on the general pattern of oviposition. Only in the cases of *C. granaria* (Eastham & McCully, 1943) and the small race of *C. oryzae* (Birch, 1945*a*) have full data been obtained under adequately controlled conditions.

In *C. oryzae* Richards (1944) has demonstrated that there are two very distinct strains which differ principally in size, and one of these, the small strain, has already been the subject of full investigation (Birch, 1945*a*, *b*). In the present work, therefore, attention has been mainly directed towards the behaviour of the large strain, a limited number of parallel experiments being carried out with the small weevil for comparative purposes.

TECHNIQUE

The original stocks of the two races of *C. oryzae* were kindly supplied by Dr O. W. Richards from the field station of the Imperial College of Science and Technology at Slough, Bucks. From these, stock cultures were set up and maintained as detailed elsewhere (Eastham & McCully, 1943; Eastham & Segrove, 1947), except that as

C. oryzae proved to be more susceptible to humidity than *C. granaria* it was necessary to ensure that grain was sufficiently moist before being supplied to the weevils. Accordingly, the soft English wheat, which was used throughout the experiments, was packed in stoppered jars in which were also enclosed gauze-covered jars containing a quantity of saturated salt solution (in equilibrium with an atmosphere of 70% R.H.). By storage in this way for a week or two the wheat was brought to a moisture content suitable for the development of the weevils. No further precaution was taken other than keeping the culture jars lightly covered to prevent undue drying out. The weevils used for infecting the stock culture were left in contact with the wheat for a period of approximately 3 weeks and were then removed by sieving and sorting. Cultures were kept going continuously at 20 and 25° C., and the weevils reared at each of these temperatures were used only in experiments carried out at the same temperature as that at which they had been reared. All the experimental insects were thus the progeny of insects which for a number of generations had been reared under constant-temperature conditions. By this means was excluded any possibility of variation in the behaviour of individual insects in any particular experiment being caused by variations of temperature in the environment of the previous generations.

When the adult weevils began to emerge from the grain the stock cultures were carefully sieved and sorted daily, and the daily emergence groups were matured separately for a period of 3–5 days before being used in experiments. The groups were then sexed by examination of the rostrum under the binocular microscope, the coarsely punctured rostrum of the male being easily distinguished from the more sparsely punctate structure of the female (Richards, 1947). No failures were subsequently recorded as a result of relying solely on this method.

All the experiments were carried out with single pairs of weevils or with isolated females. In all cases at least fifteen replicates were set up, a procedure amply justified by the results, since the performance of individual weevils varied greatly. The insects were placed in $2 \times \frac{1}{2}$ in. glass tubes which were closed with cotton gauze held in place with rubber bands. They were supplied with a certain number of grains of wheat previously acclimatized to a particular humidity by the methods outlined by Eastham & Segrove (1947). The tubes were stacked in glass jars with tightly fitting lids containing potash solutions as humidity controls (Buxton & Mellanby, 1934), which were then transferred to constant-temperature rooms and incubated in the dark at the appropriate temperature. Every 14 days the weevils were supplied with fresh wheat in clean tubes, and this procedure was continued until all the female weevils had expired. Where pairs of weevils were being used and the male died first it was replaced by a male from the same age group. The used wheat was incubated for a further 14 days at 70% R.H. in order to permit the eggs to hatch out and thus to facilitate the subsequent counting, and was then stored in 50 or 70% alcohol according to the water content of the grain, the harder grain being stored in the weaker alcohol. After storage in this way the grain was of a soft cheesy consistency, and the number of contained larvae was determined by carefully slicing the grain under the binocular microscope with a razor blade.

EXPERIMENTAL RESULTS

Experiments were carried out at two temperatures and two humidities, viz. 20 and 25° C. and 50 and 70% R.H. The choice of the temperatures was dictated solely by the limitations of the available apparatus; in particular, it was found impracticable to employ temperatures below 20° C., the duration of many of the experiments precluding their restriction entirely to the colder months of the year. Regarding the humidities, *C. oryzae*, as previously mentioned, is more susceptible to moisture than *C. granaria*; it survives for a period of days only at 40% R.H., and even at 50% R.H. mortality is high and the duration of life short. The upper limit of humidity, i.e. 70% R.H., was dictated by the difficulty of obtaining acclimatized wheat clear of fungal infection at any higher humidity.

(1) *Effect of temperature and humidity on the fecundity of the large strain of* Calandra oryzae

Four sets of fifteen replicates of pairs of weevils were set up, one set for each combination of the temperatures and moisture conditions employed, viz. 20 and 25° C. and 50 and 70% R.H. Each pair of weevils was supplied fortnightly with twenty fresh grains of acclimatized wheat. The mean number of eggs produced per female under the various conditions was as follows:

	50% R.H.	70% R.H.
20° C.	44·6 ± 4·6	235 ± 17·4
25° C.	37·1 ± 4·7	217 ± 19·7

The mean rates of oviposition are shown graphically in Fig. 1. In calculating the means it has been thought advisable to reject certain results. Commonly in each experiment one or two females produced an abnormally low number of eggs, and though the cause of this has not been determined there is every likelihood that a proportion of insects was damaged by handling, even though this was reduced to a minimum. For instance, the total numbers of eggs produced by the individual weevils at 20° C. and 70% R.H. were as follows: 325, 323, 308, 289, 276, 245, 232, 208, 204, 192, 171, 146, 137, 76, 65. The last two figures are suspiciously low, and examination of the full tabulated results shows that the weevils concerned were short-lived and had low rates of oviposition. Such results have been rejected, and though the method adopted is necessarily somewhat arbitrary it is believed that the figures given are as a result less biased than would otherwise be the case. It may be noted, however, that the rejected results have invariably differed significantly from the mean of the remainder, taking $P = 0{\cdot}05$ as a test of significance. Throughout the paper the means have been expressed in terms of their standard errors calculated according to the formulae of Simpson & Roe (1939).

From the results it is clear that the general pattern of the insect's behaviour is the same throughout the experiment. In each case the rate of oviposition rises rapidly to a peak, which is reached between 4 and 6 weeks after the emergence of the adult

weevil from the grain. It then gradually declines, becoming zero about a week before the death of the weevil, the oviposition period being thus slightly shorter than the life-span of the imago. The apparently steady decline is, however, to some extent an artefact, due to averaging a series of weevils with varying life-spans. Individual weevils show a 'middle' period, following the peak, in which the rate of oviposition falls slowly, followed by a terminal period in which the decline is rather more abrupt.

Despite this general similarity in behaviour both humidity and temperature have important effects on oviposition. It is abundantly clear that 50% R.H. is approximating to the insect's limit of viability, for at this humidity the weevil's life (and therefore the oviposition period) is short, the rate of oviposition is low and hence fecundity is at a low level. At the higher humidity the weevil lives for a longer

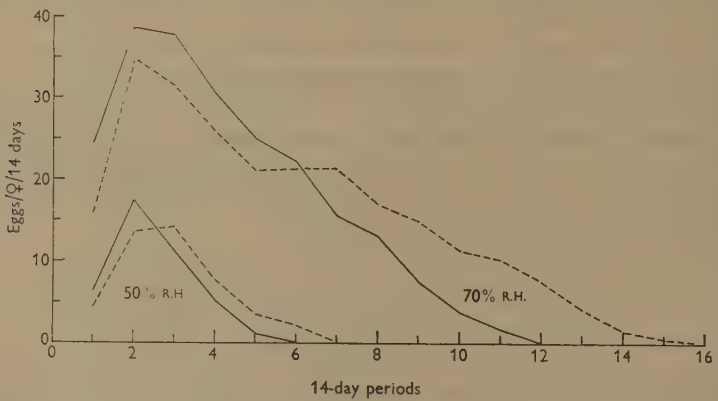


Fig. 1. Oviposition rates of paired females of the large strain of *C. oryzae* in 20 grains of wheat at different temperatures and humidities. – – – 20° C. (50% and 70% R.H.); ——— 25° C. (50% and 70% R.H.).

period and the rate of oviposition is considerably greater. It is noteworthy that temperature has little effect on the total egg production, for though the number produced at 25° C. is slightly less than at 20° C., both at 50 and 70% R.H., the differences are not significant. Temperature does, however, affect the rate of oviposition which is higher at 25 than at 20° C. at either humidity. The unaltered egg production at the higher temperature is thus due to a higher rate of oviposition coupled with a shorter oviposition period.

(2) *Effect of grain supply on fecundity in the large strain of* Calandra oryzae

Four sets of fifteen replicates of pairs of weevils were set up at 20° C. and 70% R.H. and supplied fortnightly with 5, 10, 20 and 40 grains of wheat respectively. The mean egg production of the four sets was: 5 g., 141·2 ± 7·6; 10 g., 156·3 ± 12·7; 20 g., 235 ± 17·4; 40 g., 269 ± 13·4. The differences between the means of the five- and ten-grain sets and between those of the twenty- and forty-grain sets are not significant, but all other differences are highly significant. It is quite clear, therefore,

that the amount of available grain has a pronounced effect on the fecundity of the insect, higher egg production being associated with greater amounts of grain. The rates of oviposition are graphically expressed in Fig. 2, from which it may be seen that with five, ten or twenty grains of wheat available there is little variation in the behaviour of the insect. As in the preceding experiment oviposition rises rapidly to a peak and subsequently declines gradually to the end of the oviposition period. With forty grains of wheat available, however, the oviposition period is appreciably shorter, as also is the life of the insect, and the decline in the rate of oviposition is accordingly more rapid. It may be tentatively concluded that a high fecundity coupled with a high rate of oviposition has a tendency to shorten the life of the imago.

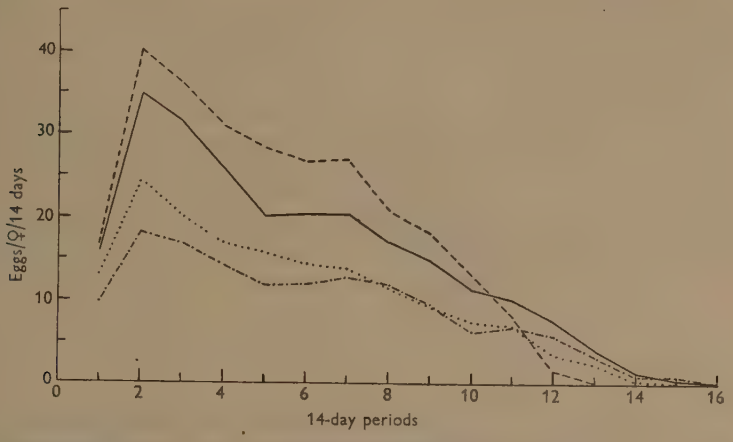


Fig. 2. Oviposition rates of paired females of the large strain of *C. oryzae* at 20° C. and 70% R.H. in different quantities of wheat. – – –40 grains; ——— 20 grains; . . . .10 grains; · – · – 5 grains.

(3) *Effect of males on fecundity in the large strain of* Calandra oryzae

Twenty replicates of pairs of weevils were set up at 25° C. and 70% R.H. and supplied fortnightly with twenty fresh grains of wheat. At the end of the first fortnight the males were removed from ten of the replicates, so that from then onwards the females were in isolation. The remaining males were removed from the other replicates at the end of the second fortnight. The mean egg production of the whole set of weevils was $247{\cdot}2 \pm 9{\cdot}8$. This figure is higher than that for pairs of weevils supplied with twenty grains of wheat under similar conditions (i.e. $217{\cdot}6 \pm 19{\cdot}8$), but the difference is not significant. Comparison of the rates of oviposition, however, shows a striking difference. From Fig. 3 it can be seen that in the case of the isolated females the rate of oviposition rises steeply to a high peak, again somewhere between the 4th and 6th weeks of oviposition, then rapidly falls away and ceases several weeks earlier than happens with the paired weevils. Thus though the isolated females do not produce a significantly greater number of eggs than the females paired with males they do deposit them in a considerably shorter space of time and exhaust themselves

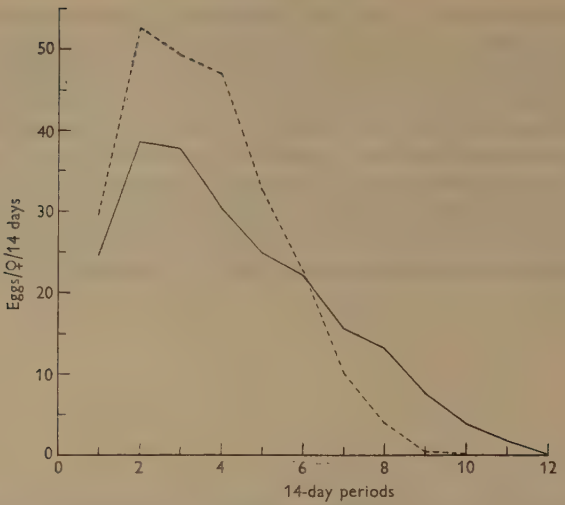


Fig. 3. Comparison of oviposition rates of paired and isolated females of the large strain of *C. oryzae* in 20 grains of wheat at 25° C. and 70% R.H. ——— Paired females; – – – isolated females.

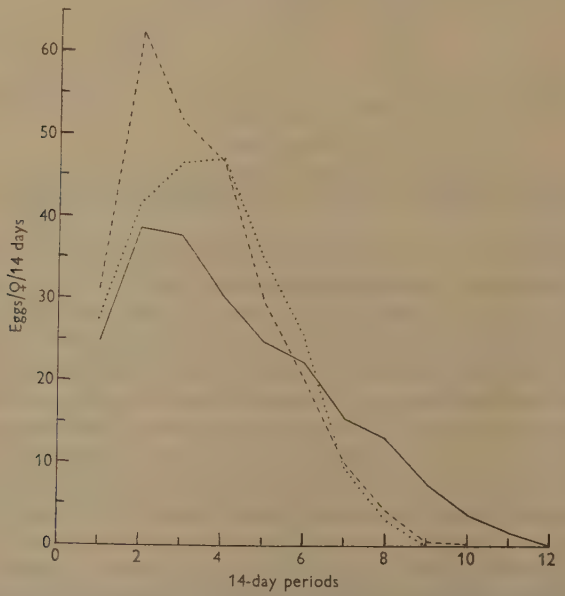


Fig. 4. Comparison of oviposition rates of paired and isolated females of the large strain of *C. oryzae* in 20 grains of wheat at 25° C. and 70% R.H. ——— Paired females; – – – females isolated after 2 weeks; · · · · females isolated after 4 weeks.

much sooner. This more rapid rate of oviposition is accompanied by a somewhat shorter life-span. These effects are still more striking if the two groups of isolated females are considered separately (see Fig. 4). In the case of the females isolated at the end of the first fortnight, the rate of oviposition rises to a maximum at the end of 4 weeks, when it is more than 50% higher than that of the paired females. From this peak value it declines steadily. In the case of the females isolated after a month it can be seen that the rate of oviposition follows the expected course for the first 4 weeks (i.e. parallel to, though slightly higher than, the course followed by the permanently paired females), but that subsequent to the removal of the males the rate of oviposition continues to rise for a further 4 weeks before declining. The maximum rate of the second batch of females is considerably less than that of the first batch. As a result of this, maximum egg production is shown by the females isolated after 2 weeks, whereas those isolated after a month show a somewhat lower fecundity coupled with a rather different pattern of oviposition behaviour. These results appear strongly to indicate that the females possess, in the first few weeks of adult life, a very high egg-laying potential which can be realized only in a relatively short period. Any factor, such as in this case the presence of males, which tends to restrain the rate of oviposition and which is operative during this period has a permanent effect on the rate of oviposition, and possibly on the fecundity. Removal of the restraining factor after even a relatively short period (i.e. in this case 2 weeks) is not compensated by a subsequent rise in the rate of oviposition to the value which would have been reached in the early stages. Compensation does, however, occur and takes the form of an extension of the oviposition period. The difference between the two sets of isolated females in this respect is not great, but if either is compared with the permanently paired females the effect of the initial depression of the rate of oviposition on the overall pattern of oviposition is clearly indicated. It is not possible to say whether the maximum egg-laying potential has been realized in these experiments, since no data is available for the performance of isolated females in larger quantities of grain. It does seem rather unlikely, however, from the results of other workers. MacLagen & Dunn (1936) record a maximum rate of oviposition of 6·75 eggs/female/diem at a density of 1 weevil to 400 grains, as compared with a present maximum of 4·5 eggs/female/diem. Hinds & Turner (1911), according to these same authors, state that in maize a value of 15–16 eggs/female/diem is not uncommon. Neither of these results, however, must be interpreted as indicating that the total fecundity would exceed the values recorded here by anything like the same margin.

In the present experiments there was no appreciable increase in the number of infertile eggs at the end of the oviposition period. It does not therefore seem likely that the rapid falling off in the rate of oviposition observed is connected in any way with the exhaustion of sperm in the spermatheca of the female, though, unfortunately, this point was not checked. In some insects, e.g. *Rhizopertha* (Crombie, 1942), it is known that a single or several early impregnations are sufficient for unrestricted oviposition, subsequent copulations having no influence on fecundity or fertility. In *Calandra* such evidence as is available (Richards, 1947) indicates that in general

the copulation of females which have been in isolation for considerable periods has little effect on oviposition. It has, however, been observed in the course of the present work that in most females the stimulus of copulation is necessary to initiate oviposition.

(4) *The relative fecundity of the two strains of* Calandra oryzae

Oviposition in the small race of *C. oryzae* was investigated only at 25° C. At this temperature and at a R.H. of 70% the mean number of eggs produced by 15 replicates of pairs of weevils supplied fortnightly with 20 grains of wheat was $148{\cdot}8 \pm 13{\cdot}7$. This differs significantly from the number of eggs produced by the large strain under similar conditions, viz. $216 \pm 19{\cdot}7$, and agrees with the findings of other workers on

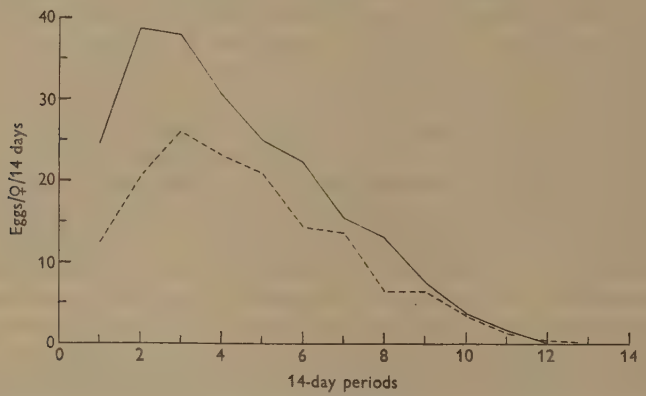


Fig. 5. Comparison of oviposition rates of the two strains of *C. oryzae* in 20 grains of wheat at 25° C. and 70% R.H. ——— Large strain; – – – small strain.

the relative activity of the two strains (see Richards, 1944). There is little difference in the life-span of the two strains, and the lower egg production of the small strain is therefore attributable to a lower rate of oviposition, as may be seen by reference to Fig. 5, in which the rates of oviposition of the two strains are compared. Birch (1945*a*) records the figure of 265 for the egg production of the small strain at 25·5° C. and 70% R.H. with a weevil density of 1–10 grains of wheat, but in his case the grain was changed weekly. The extent to which this latter factor might explain his much higher figure is discussed in a later section.

(5) *The effects of grain size on the distribution of eggs in grain in the two races of* Calandra oryzae

During the counting of eggs in the foregoing experiments the numbers in individual grains were recorded in full. Examination of these results appeared to indicate that the distribution of eggs differed in the two strains of weevil, further analysis confirming this impression and yielding some evidence in explanation of the different behaviour of the two strains.

If a number of eggs are deposited at random in a given number of wheat grains the probabilities of obtaining grain containing 0, 1, 2, etc., eggs may be calculated from the expansion of the binomial $\left(\frac{n-1}{n}+\frac{1}{n}\right)^r$, where n is the number of grains and r the number of eggs. As, in general, a few terms only are required the formula ${}^{n^r}C_x\left(1-\frac{1}{n}\right)^{r-x}\left(\frac{1}{n}\right)^x$, given by Stoy (in appendix to Salt, 1932) and derived from the general term of the expansion, may be conveniently used. This gives the probable number of grains of the total number of n which will contain x eggs when the total number of eggs deposited is r, and is calculated for values of $x=0, 1, 2, \ldots$, etc. For instance, the random distribution of 10 eggs in 20 grains gives the following values:

No. of eggs per grain	0	1	2	3	4 or more
Frequency	11·97	6·30	1·50	0·21	0·02
No. of eggs × frequency	0	6·30	3·00	0·63	0·08

At 25° C. and 70% R.H. weevils of the small strain, during the course of the experiment, laid between 9 and 11 eggs in 20 grains in a 14-day period on six occasions; weevils of the large strain under the same conditions did likewise on five occasions. The numbers of grains containing 0, 1, 2, etc. eggs from the aggregates of these results are shown in Table 1, together with the expected frequencies of random distribution calculated from the table above. (The figures for the small strain have been reduced by one-sixth in order to equate them to those for the large strain.) The agreement between the three sets of figures is extremely close and would seem to indicate that at this rate of oviposition (i.e. 10 eggs/20 grains/14 days) both strains of the weevil deposit eggs at random. It is, nevertheless, doubtful whether these results are anything more than fortuitous, for at higher intensities of oviposition a pronounced difference in the behaviour of the two strains becomes apparent.

Table 1

Eggs per grain	0	1	2	3	4 or more
No. of grains (large strain)	59	33	7	1	0
No. of grains (small strain)	59·2	32·5	7·5	0·8	0
Expected frequency on random distribution	59·85	31·5	7·5	1·05	0·1

The distributions of 250 eggs in 200 grains by each type of weevil (aggregates in each case of 10 cases where 24–26 eggs were laid in 20 grains in a fortnight) are shown in Table 2 and depicted graphically in Fig. 6. It may be seen that in the case of the small strain there are fewer 'empty' grains, more grains with one egg, and fewer with two or more than is to be expected on random distribution, while the reverse is the case with the large strain. In neither case, therefore, are the eggs distributed at random. Similar results are obtained with the cases in which 20 eggs were laid in 20 grains.

Table 2

Eggs per grain	0	1	2	3	4	5 or more
No. of grains (large strain)	70	55	42	24	6	3
No. of grains (small strain)	38	101	42	12	6	1
Expected frequency on random distribution	55·5	73·0	46·1	18·6	5·4	1·4

The 'over-distribution' by the small weevil appears to indicate a tendency on the part of the insect to avoid grain in which eggs have already been deposited or in which larvae are present. This conclusion will be discussed later in relation to the

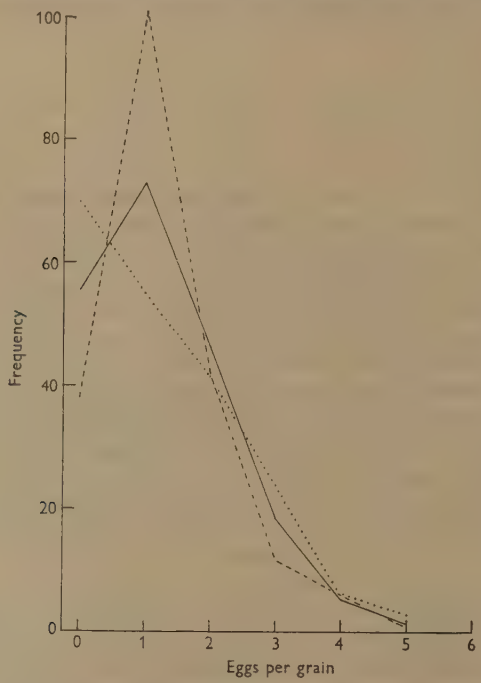


Fig. 6. Distribution of eggs in grain by the two strains of *C. oryzae*. Data from ten cases, for each strain, in which 24–26 eggs were laid in 20 grains in 14 days. · · · · Large strain; – – – small strain; ——— random distribution.

results of other workers. The 'under-distribution' by the large strain seems, on the other hand, to be correlated with the size of the wheat grains and the tendency for the weevil to select the larger grain for oviposition. This has been demonstrated by the examination of one fortnightly batch of 300 grains supplied in the usual way to 15 weevil pairs in lots of 20. Previous to the counting of the eggs by the slicing technique each individual grain was weighed; any grains showing more than slight

signs of being eaten were rejected and the remainder were divided into heavy and light halves. From the aggregates of the heavy and light halves the following figures were obtained: the 134 heavier grains contained 321 eggs, 13 grains containing no eggs; the 134 lighter grains contained 189 eggs, 42 grains containing no eggs. This result is, of course, partially explicable in terms of the larger surface area available for oviposition in the heavier grains. Calculation shows, however, that after due allowance is made for this factor a disproportionately large fraction of the total eggs occurs in the heavier grains, whilst the discrepancy is even more apparent in the distribution of the empty grains. It is evident that the weevil prefers the heavier, and therefore larger, grain for oviposition. In addition, calculation indicates that in the heavier grain the distribution of eggs is at least as good as random with the possibility of over-distribution (as found in the small strain), though the numbers involved are too small to be conclusive. It appears, therefore, that the difference between the results with the two strains is related to their preferences for different sizes of grain and that the average size of the experimental grain fell below the optimum of the large strain. It should be added that the suitability of grain for oviposition is determined by its shape as well as by its size or weight. Grains with a characteristic plump shape are avoided, whatever their size, as meticulously as the very small ones, though the reason for this avoidance is not clear.

ANALYSIS OF RESULTS AND DISCUSSION

One of the most striking features of the results in general is the great variation in the performance of the individual weevils. This in part is doubtless to be attributed to genetic variation in the stock and can be dealt with only by conducting experiments on the largest possible scale if statistically significant results are to be obtained. Experience has shown that it is not possible to circumvent this problem by selecting weevils showing an average performance over a short period, a practice frequently employed in short-term experiments. Fecundity in the early weeks of adult life is no sure index of total egg capacity, low initial fecundity being followed on occasion by maintained oviposition, high initial fecundity by rapid decline and a relatively low egg total. A further difficulty arises when the procedure adopted in the present work is employed, viz. of rearing stocks at the temperatures it is intended to apply during the experimental work. In such circumstances it is impossible to test the females under identical conditions before commencing the experiments, and careful determinations of average performances under different conditions would have to be made if the final results were to be in any way comparable. In view of these facts it is clearly desirable to eliminate, as far as possible, the contribution of the environment to the induction of variability in the stock. Two points seem worthy of note. In the first place Richards (1944) has shown that grain size influences the size of the adult insect, and also (1947) that egg production is related to the size of the weevil. A more uniform size of weevil and therefore a more uniform oviposition performance may be expected if grain of a standard size is used in stock cultures. In the second place it seems desirable to avoid the use of mass cultures in rearing the experimental

insects. In such cultures accumulation of carbon dioxide or frass and overheating in the interior of the mass are all factors calculated to produce non-uniformity, whereas if the grain were spread out in thin layers, say in desiccators, such effects could be entirely eliminated.

From the results of the individual experiments a number of points emerge which require brief discussion. The effects of temperature on oviposition will first of all be considered. In an earlier paper (Eastham & Segrove, 1947) it was shown that an insect could be subjected to approximately equivalent moisture conditions at several different temperatures by employing throughout a fixed relative humidity, and that by so doing it is possible to isolate the effects of temperature as an environmental factor. In the present work this has been done (see § 1 above), and the effect has been to demonstrate that there is no significant difference between the total eggs deposited at temperatures of 20 and 25° C. under equivalent moisture conditions. It may be concluded that the main effect of temperature changes is to alter the general tempo of the life of the insect. The results of Eastham & McCully (1943) on oviposition in *C. granaria* are in broad agreement with this conclusion if due allowance is made for variability in the weevil stock on a similar scale to that encountered in the present work.

The second point to be considered concerns the distribution of eggs between grains. The results of the present work have established with reasonable certainty that the small strain of *C. oryzae* exhibits a marked tendency to avoid laying eggs in grain already containing eggs and/or larvae, and that though the large strain of weevil exhibits an apparent inverse effect, i.e. tends to overcrowd its eggs, the result is to be ascribed to its greater preference for a larger-sized grain, and its behaviour in reality is no different from that of the small strain. Moreover, although it has not been established that there is an optimum size of grain for each weevil, since it was not possible to show that the smaller weevil was not also making more use of the larger grain, it is nevertheless clear that the smaller weevils utilize the smaller grain far more readily than the larger weevils. The most likely explanation for this is that the females of the larger strain have more difficulty in manipulating the small grain in the act of oviposition. This point is of some interest, since Ewer (1945), working with *C. granaria*, failed to establish any substantial difference in the performance of selected small and large female weevils when presented with unselected grain. Both showed a preference for large grains, and the smaller weevils showed no less preference than the larger ones, and Ewer concludes that the behaviour of the animals is influenced by physico-chemical differences in the grain rather than by size. This author also showed that the female weevils tend to avoid laying in grain containing a fourth instar larva, but otherwise found no evidence of oviposition being inhibited by the presence of eggs or larvae. One of her experiments, quoted in more detail by Richards (1947), does nevertheless support this idea.

In this particular experiment a mixture of large and small grains—the proportion of each being such that the total surface areas of each size of grain were equal—were presented for a short period to a group of weevils. In the subsequent analysis it was found that more eggs had been laid in the large grains than in the small, and that

there were fewer grains with 0 or 2 eggs per grain and more with 1 or 4 than could be expected on random distribution. Richards contends that the abnormal distribution is due to the admixture of two sizes of grain 'in either of which alone oviposition would have been normal', i.e. random. I believe, however, that it is a clear case of over-distribution of eggs, and that even the slight excess of 4-egg grains is explicable if a somewhat different method of analysis is employed. Strictly speaking it is permissible to compare the actual distribution of eggs with the calculated random distribution only when the eggs are partitioned between the two types of grain in proportion to the numbers of grain of each type (or alternatively in proportion to their aggregate surface areas), and this is manifestly not the case in Ewer's experiment. An alternative procedure is to make separate calculations of the random distribution for the two types of grain and compare the sums of the two sets of results with the experimental data. It is not difficult to show that this second method is the better one to apply in the present case.

The actual experimental situation lies somewhere between two clearly defined limiting situations, which are: that the weevils either show an equal preference for the two types of grain, or, at the other extreme, confine their attention (as regards oviposition) strictly to one type or the other. In the former case it would be correct to determine the random distribution for the whole grain sample, but in the latter contingency the calculation should concern only that fraction in which the eggs are laid, i.e. the two types of grain should be treated quite independently in the calculation. An appreciation of this point is of fundamental importance to the whole argument, for the two sets of values are widely different, as comparison of lines 1 (or 2) and 3 in Table 3 will show. My contention is essentially that in the second

Table 3. *Distribution of* 164 *eggs in a mixture of* 185 *small grains and* 116 *large grains*

	Frequencies of grain containing (eggs per grain)				
	0	1	2	3	4
Random distribution of 164 eggs in 301 grains	174·4	95·3	25·9	4·7	0·6
Sum of random distributions of 63 eggs in 116 grains and 101 eggs in 185 grains	174·2	95·6	25·9	4·6	0·5
Random distribution with all (164) eggs in large (116) grains	213·0	40·0	28·3	13·3	6·4
Sum of random distributions of 102 eggs in 116 grains and 62 eggs in 185 grains	180·2	86·8	26·0	6·2	1·8
Distribution in actual experiment	172*	105	16	5	3

Lines 1–4 show the calculated random distributions for different circumstances (see text for explanation). The experimental data in line 5 are from Ewer (1945) and Richards (1947).

* The figure 160 which occurs in Richards' table at this point is apparently due to an error in recording the number of large grains containing no life. As a further check, however, his detailed data *as given* have been analysed as in the present table and the results are entirely in agreement with those presented here.

limiting situation the environment of the insect is divisible into two distinct sub-environments, only one of which is being utilized for oviposition and which therefore should be treated of as a separate entity as regards this particular activity. However,

once the insect begins to utilize the other type of grain the two sub-environments begin to overlap or coalesce, and do so completely when the insect ceases to discriminate between the two sizes of grain. Hence it would appear that to subject the two sub-environments to separate analysis in such intermediate situations would inevitably be more or less incorrect. One could obviously make an estimate of the relative importance of the two parts of the environment from the partition of eggs between them and then decide to which limiting situation the experimental situation more closely approximated in order to choose the better method of analysis. Fortunately this is unnecessary, for the position is far more precise than appears on the surface. The order of the numbers involved in the present case is such that even the hypothetical situation where both sorts of grain are equally acceptable to the weevils, i.e. where there is a single uniform environment, may be analysed in two separate parts with little loss of accuracy. This will be apparent from a comparison of the two sets of values in lines 1 and 2 in the table, which have been arrived at in the following manner. Line 1 is a straightforward evaluation of the random distribution for the total eggs in the total grain. In line 2 the total of 164 eggs has been divided between the large and small grains in proportion to the numbers of each type of grain present, and yields values of 63 eggs to the 116 large grains and 101 eggs to the 185 small grains. Separate evaluations of the random distributions for each type of grain have then been made, and the two sets of values have been added together to obtain a single set of figures for the whole of the grain. The figures obtained by the two methods of calculation are almost identical (see lines 1 and 2 in the table). This means, in effect, that even when the insect is showing no discrimination whatever, independent analysis of the two types of grain introduces a negligibly small error. Furthermore, this error reduces to vanishing point by this method of treating the results as the experimental situation approaches the other extreme where the insect makes exclusive use of one type of grain.

The random distribution for the experimental result has therefore been evaluated by calculating separately the random distributions in each type of grain in terms of the numbers of eggs actually laid in each type, and then summing the corresponding figures. Thus since 101 eggs were laid in 116 large grains and 62 eggs in 185 small grains in the actual experiment, these two pairs of figures have served as the basis for the calculation of two random distributions. The values obtained from the sum of these distributions are shown in line 4 of the table. Comparison of them with the experimental results in line 5 reveals appreciable differences. The numbers of grains with more than two eggs are too small to be significant, but the excessive number of 1-egg grains in the experiment, and the corresponding deficiency of empty and 2-egg grains, leave little room for doubt that the eggs are not deposited at random. One can only conclude from this more effective distribution that the weevils exhibit a definite tendency to avoid grains in which eggs have already been deposited.

This conclusion, if the validity of the argument be admitted, is apparently at variance with other of Richards (1947) data. For example, in a detailed analysis of five cultures in unselected wheat he finds that eggs are distributed at random (though a closer inspection indicates that the result actually shows slight under-

distribution). Elsewhere, by plotting percentage grain attacked against life per 100 grains, for a large number of cultures, he produces further evidence of random distribution. Both results, however, are explicable in the light of the evidence from the experiment analysed above and the results given earlier for the large strain of *C. oryzae*, viz. of the existence of two opposing tendencies in the weevil—to distribute the eggs widely by avoiding grains with life on the one hand, to crowd the eggs into the more suitably sized grains on the other. Under certain circumstances, when the grain available to the weevil is of some particular average size, these opposing factors balance one another. The resulting distribution is superficially a random one, and its spurious character is concealed rather than made evident by certain methods of analysis. It is clearly necessary to distinguish between a true 'random distribution' due to genuine random behaviour on the part of the insect and an 'effectively random distribution' which may arise from other causes. It is quite feasible, and I think probable, that these points provide an explanation of Richards's results, and that further investigations in which use is made of grain of different sizes will confirm the findings of the present paper.

MacLagen & Dunn (1936), also working with *C. oryzae*, are the only workers who appear to have suspected that the weevil is capable of detecting and avoiding grain containing life, but their reasons for doing so are somewhat obscure. Using a series of different densities of weevils/grain they found that the frequency curve of egg distribution altered from a marked positive skewness at low densities to something approaching a normal curve at high densities. They suggest that whilst the former may indicate what I have termed 'over-distribution', the latter undoubtedly means that the weevils use the grain indiscriminately at high densities. I can agree with neither finding. Marked skewness of the frequency curve at low concentrations of eggs/grain is not of itself any proof that the distribution is other than random, as reference to Fig. 6 in the present paper will show. In the second place, the frequency curve automatically becomes less skew and approaches normality as more and more eggs are crowded into a given number of grains, and it becomes increasingly important to show that the deviation from a normal distribution is statistically significant before any reliable conclusion can be drawn.

The main reason for this attempt to establish the existence of a non-random behaviour on the part of the weevil in its choice of oviposition sites is that if proved it becomes yet another factor to be considered in the interpretation of experimental results. For if the insect is influenced to deposit eggs in other grains by the presence of eggs or larvae, the occurrence of these in appreciable numbers seems likely to depress the rate of oviposition. This may, for instance, be a partial explanation of the discrepancy between Birch's (1945*a*, *b*) results with the small strain of *C. oryzae* and my own, to which previous reference has been made. Birch's figure for the fecundity of the weevil under the same conditions of temperature and humidity and the same density of weevils/grain as employed in the present work, is nearly double that recorded here. Whilst this may be due to a variety of causes—genetic differences in the stock, different strains of wheat, etc.—the mere fact of his changing the wheat with greater frequency, actually weekly instead of fortnightly, may have contributed

substantially to the production of the higher figure. Not the least effect of this difference in technique would be to relieve the congestion of grains by eggs, and thus to ameliorate a condition likely to inhibit oviposition. Alternatively, of course, the rate at which this congestion builds up can be reduced by increasing the quantity of grain. The present series of experiments, and those of MacLagen & Dunn (1936), have shown how fecundity rises as more grain is made available (vide § 2 above). In part, this is undoubtedly to be explained by the reduced probability of the male disturbing the female in the act of oviposition in a larger volume of grain (as the experiments which isolated females clearly emphasize), but may also be due to the effect under discussion. More intensive experiments with isolated females in different quantities of grain would probably enable the relative importance of the two effects to be determined; the present data, unfortunately, is insufficient to decide the point. It may be added that such experiments would also shed some light on the results of MacLagen & Dunn (1936). They found that a maximum rate of oviposition was obtained with a density of weevils/grain of 1 : 400, but that under these conditions only 1 grain in 11 was used in oviposition.

Finally, a brief reference must be made to a second important point which emerges from the experiments with isolated females. The marked rise which occurred in the rate of oviposition in these experiments following the removal of the males was found ultimately to be unaccompanied by any statistically significant increase in the total eggs laid. This serves to emphasize a danger which is inherent in short-term experiments, viz. of translating the short-term effects of changed conditions into terms of an overall change in performance. Where, therefore, an accurate knowledge of the fecundity is required, as, for example, in the computation of the 'biotic potential' of a population (see Birch, 1945*b*) there is no alternative to the complete investigation of the insect's reproductive span.

SUMMARY

The oviposition behaviour of the large strain of *Calandra oryzae* has been investigated at 20 and 25° C. and under moisture conditions equivalent to 50 and 70% R.H. The small strain of the weevil has been investigated at 25° C. and 70% R.H.

At either temperature the lower humidity shortens the life of the insect and depresses the rate of oviposition so that fecundity is of a low order. Changes of temperature with a fixed humidity alter the rate of oviposition but have little effect on the total egg production. Under all conditions the pattern of oviposition remains the same, the oviposition rate rising to a peak in the earlier weeks of maturity and subsequently declining.

Increasing the amount of grain leads to increasing egg production. It is unlikely that conditions for maximum fecundity were realised in the experiments.

Isolating females in the early weeks of maturity leads to a high initial rate of oviposition, followed by a more rapid decline and little overall increase in fecundity.

At 25° C. and 70% R.H. the fecundity of the large strain is of the order of 50% higher than that of the small strain.

The distribution of eggs between grains differs in the two strains, the small strain distributing its eggs better than random, the large strain tending to overcrowd its eggs. The evidence suggests that this is due to the larger weevils' greater preference for large grains. By correlating the results with those from other sources it is nevertheless concluded that both strains tend to avoid laying in grains already containing life. The importance of this factor in oviposition behaviour is discussed.

REFERENCES

Birch, L. C. (1945*a*). *Aust. J. exp. Biol. Med. Sci.* **23**, 197.
Birch, L. C. (1945*b*). *Trans. Roy. Soc. S. Aust.* **89**, 140.
Buxton, P. A. & Mellanby, K. (1934). *Bull. Ent. Res.* **25**, 171.
Chapman, R. N. & Baird, L. (1934). *J. Exp. Zool.* **68**, 298.
Crombie, A. C. (1942). *J. Exp. Biol.* **19**, 311.
Eastham, L. E. S. & McCully, S. B. (1943). *J. Exp. Biol.* **20**, 35.
Eastham, L. E. S. & Segrove, F. (1947). *J. Exp. Biol.* **24**, 79.
Ewer, R. F. (1945). *Proc. Ent. Soc. A*, **20**, 57.
Hinds, W. E. & Turner, W. F. (1911). *J. Econ. Ent.* **4**, 230.
Kunike, G. (1936). *Z. angew. Ent.* **23**, 303.
Lavrekhin, F. A. (1937). *Bull. Soc. Nat. Moscou*, Sect. Biol., **46**, 225.
MacLagen, D. S. & Dunn, E. (1936). *Proc. Roy. Soc. Edinb.* **55**, 126.
Richards, O. W. (1944). *Trans. Ent. Soc.* **94**, 187.
Richards, O. W. (1947). *Proc. Zool. Soc. Lond.* **117**, 1.
Salt, G. (1932). *Bull. Ent. Res.* **23**, 211.
Simpson, G. G. & Roe, A. (1939). *Quantitative Zoology*. New York.

THE COLOUR CHANGE OF THE MINNOW (*PHOXINUS LAEVIS* AG.)*

I. EFFECTS OF SPINAL SECTION BETWEEN VERTEBRAE 5 AND 12 ON THE RESPONSES OF THE MELANOPHORES

By E. G. HEALEY

From the Department of Zoology, University College of Wales, Aberystwyth

(*Received 7 December* 1950)

(With Eleven Text-figures)

I. INTRODUCTION

(*a*) *Previous work*

It is now well established that melanophores in teleosts are controlled by both nervous and humoral mechanisms. Sympathetic nervous control was shown to exist by Pouchet (1876) in the turbot and other flat fish. His results were confirmed by various workers but received their greatest extension through the work of von Frisch (1911). He showed that, in the minnow, fibres causing aggregation of the melanophores pass from a centre in the medulla along the spinal cord. At about the level of the 15th vertebra these fibres enter the sympathetic chain and there run forwards and backwards, passing out with the spinal nerves to supply the melanophores of the body and with a branch of the trigeminal nerve to supply those of the head. Von Frisch found that when he cut through the fibres of this system the region of the body separated from the centre in the brain quickly became dark and could no longer take part in the normal colour changes of the fish. However, he observed that when such a fish was kept for some time upon a white background the denervated region gradually became pale; if the animal was then placed on a black background the denervated region slowly darkened again. At that time he did not pursue this matter further. Later experiments by various workers showed that melanophores could be affected by chemical substances, and Hogben and his co-workers demonstrated the role of hormones in the colour changes of Amphibia. This knowledge suggested that similar mechanisms might play a part in the colour changes of fish. That they do so has been shown by a number of workers on elasmobranchs (Lundstrom & Bard, 1932; Hogben, 1936; Parker, 1938; Waring, 1938) and teleosts (Parker, 1934; Abramowitz, 1936, 1937; Osborn, 1938; Hogben & Landgrebe, 1940; Neill, 1940; Healey, 1940; Vilter, 1941).

Smith (1931) continued von Frisch's observations on the minnow. He denervated head melanophores by cutting through the trigeminus and body melanophores by cutting through spinal nerves. He was also able to observe a slow colour change in the denervated region and showed that this depended upon an intact blood circulation. He concluded that some hormone in the blood caused the aggregation of the

* (=*Phoxinus phoxinus* L.)

melanophores and suggested that it might be adrenalin. On the other hand, Parker and his school have shown that denervated areas resulting from small transverse cuts in the tails of *Fundulus heteroclitus* and *Ameiurus nebulosus* still show some colour changes and have maintained that these are caused chiefly by neurohumours which diffuse into the denervated region from the endings of the neighbouring intact nerve fibres. The author (1940) observed the reactions of melanophores in the minnow after the spinal cord had been cut anterior to the 15th vertebra. According to von Frisch (1911) the entire peripheral chromatic nervous system should be put out of action by such an operation (as far as connexion with the brain is concerned) and the possibility of neurohumours from nerve endings playing a part should not arise. Observation showed that the fish still change their colour slowly on a white or black background, and that this colour change depends upon the presence of the pituitary gland. The latter produces a hormone which is responsible for the paling of the operated fish on a white background. There were strong indications that it may also produce a hormone which causes darkening of the fish on a black background; but the evidence for this was not conclusive.

It has been reported many times that section of chromatic nerve fibres in teleosts results in the darkening of the denervated region. Some workers have attributed this darkening to the paralysis of sympathetic aggregating fibres and others to a stimulation through cutting of parasympathetic dispersing fibres. Since the presence of aggregating fibres is not disputed there remains the possibility of a double innervation of the melanophores: a sympathetic innervation causing aggregation and a parasympathetic innervation causing dispersion. Parker has fully presented his case in favour of this view (1948). In the case of the minnow, Giersberg (1931) showed a darkening after the injection of certain parasympathetic excitants. He also found that after treatment with ergotamine followed by acetylcholine he could produce melanophore dispersion by electrical stimulation. Von Gelei (1942) carried out further experiments along these lines. After treating minnows with ergotamine and acetylcholine he cut the sympathetic chain and the spinal cord at different levels before applying electrical stimulation to the medulla. His results indicated that the fibres responsible for melanophore dispersion do not follow the same path as those responsible for melanophore aggregation as found by von Frisch (1911) but pass to the sympathetic chain from the spinal cord by the first or second spinal nerve. However, he concluded that these dispersing fibres play no significant part in the colour change of the normal animal. It is still possible that they may play a part in the colour change of minnows whose spinal cords have been cut posterior to the 2nd spinal nerve (von Frisch, private communication).

(*b*) *Present work*

In a number of papers Hogben has drawn attention to the importance of time relations in the study of chromatic responses and, from a consideration of them, he and his co-workers have arrived at a number of significant conclusions (Hogben, 1943; Neill, 1940). In the author's earlier experiments (1940) the colour changes of the minnow were estimated macroscopically by gross colour matching against

standards. All fish were tested on different backgrounds before use to ensure that they showed the same tint under similar conditions, and those which did not conform (through different sizes or concentrations of melanophores or through disease) were discarded. Nevertheless, for the timing of colour changes the method of estimating the degree of dispersion of the melanophores by the use of the melanophore index (Hogben & Slome, 1931) offers great advantages and was therefore adopted in the present work. Fig. 1 shows the various degrees of dispersion which are represented

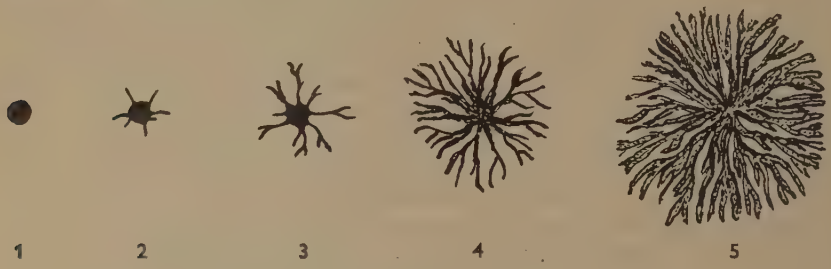


Fig. 1. The melanophore index.

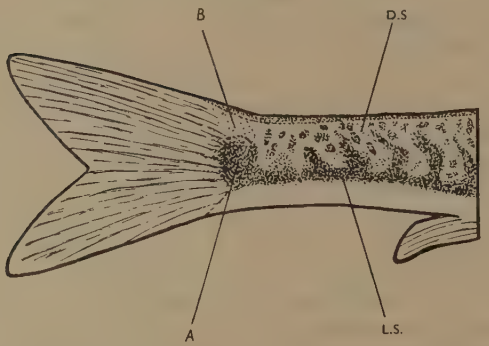


Fig. 2. Diagram of the tail region of a minnow to show the regions observed: *A* = enlarged tail region of lateral stripe; *B* = region of small melanophores; *D.S.* = small melanophores on dorsal surface; *L.S.* = large melanophores on lateral stripe.

by the M.I. values of 1–5. The minnow has both epidermal and dermal melanophores distributed on the dorsal and dorso-lateral surfaces of the body and extending to some extent into the fins. These regions are fairly sharply demarcated from the ventro-lateral and ventral surfaces on which there are few if any melanophores. The melanophores themselves are of two sizes. Large epidermal and dermal melanophores are associated in groups which macroscopically form a variably well-defined pattern. This takes the form of a thin mid-dorsal line, scattered dark flecks on the dorsal and dorso-lateral surfaces and a wide dark stripe along each side. This dark stripe will be referred to as the 'lateral stripe'. Below it the region of few or no melanophores begins. Fig. 2 shows diagrammatically the posterior region of a minnow

with the main distribution of melanophores. Those parts of the dorsal and dorso-lateral surfaces not occupied by these large melanophores are provided with very much smaller melanophores in both the epidermis and the dermis. At the root of the tail fin the lateral stripe usually becomes somewhat enlarged. This is the region *A* in Fig. 2. The region of small melanophores dorsal to the region *A* is referred to as region *B*. Readings of the M.I. were taken at *B* in the case of unoperated fish, but in the case of operated fish readings were also taken at *A* and often also on the dorsal surface (small melanophores) and the lateral stripe (large melanophores).

(*c*) *Source and general treatment of fish*

Minnows were obtained from four sources. In earlier experiments carried out in 1940 the fish came from the River Dee at Aberdeen and from the River Tweed. They were kept in the laboratory in stock tanks with a bottom of brown pebbles, supplied with running water and fed on raw finely minced meat and Cura's 'XL' fish food. In later experiments carried out at Aberystwyth in 1949 the fish were taken from the River Dovey and from Fron Gogh Pool near Trisant in Cardiganshire. These fish were kept in slate stock tanks and in light stone sinks with slate bottoms. Aberystwyth tap water, if allowed to stand, readily dissolves lead from pipes. For this reason the water was first run fast for 20 min. to ensure an uncontaminated supply from the mains. It was then collected in the tanks and treated with chalk and a rapid stream of air for 30 min. in order to reduce acidity and to remove chlorine. After these precautions had been taken the minnows were able to survive in it. They were fed upon finely minced meat and dried food supplied by Messrs Haig.

It has been shown (Healey, 1940) that minnows from different sources may differ in some of their colour change reactions. In this case no essential differences were observed in the minnows from the various sources, as far as they were studied.

Before using them for experiments fish were kept for at least a week in small glass aquaria with slate bottoms to accustom them to more restricted space and to the movements of an observer. Excitement and its consequent pallor (von Frisch, 1911; Healey, 1940) were thus largely avoided. During experiments they were usually fed little if at all in order to avoid fouling the water.

All the experiments were made in a dark room with standard illumination, namely, a 40 W. domestic electric lamp at 1 m. from the fish. The temperature of the water was maintained throughout at $12 \pm 0{\cdot}2^{\circ}$ C., unless otherwise stated.

II. THE COLOUR CHANGE OF NORMAL MINNOWS

(*a*) *General experimental procedure*

The minnows used in this series of experiments were about 3 cm. long. The containers were 250 ml. conical flasks with several holes blown in the bottom to allow rapid drainage while preventing the escape of the fish. Six of these flasks, each containing one fish, stood on glass props in 4 cm. of water in a glass aquarium. This rested upon a black or white painted metal tray with sides 7 cm. high. The tray and aquarium were immersed in a thermostatically controlled bath. A stream of air was passed through the water in each flask and through the water in the aquarium. In this way there was adequate aeration and thorough mixing of water. Since it was difficult to remove the aerator tubes quickly from the flasks without disturbing the fish, they

were cautiously taken out 20 min. before the experiment began. For the remainder of the time the fish obtained their oxygen in adequate supply from the water in the flasks and in the aquarium.

The fish were first kept for 7 days in white or black painted aquaria whose temperature was not controlled, depending upon the background adaptation required at the beginning of the experiment. The level of water in these aquaria was then carefully reduced until the fish could easily be made to swim into the flasks, i.e. all handling of the fish was avoided. After 12 hr. in the flasks on the appropriate background, arranged as described above, the experiment proper began.

When timing the colour change from white to black and from black to white backgrounds the whole aquarium with its contained flasks was quickly lifted on to a tray of the appropriate colour which was already lying in the constant-temperature bath beside it. The fish were then removed from the flasks after various time intervals and observed. This was done by lifting the flask from the aquarium (and so draining it almost as quickly as it could be removed) and tipping the fish, which almost always came out head first, on to a grid made of glass rods cemented to a glass plate. This grid method has been fully described by Neill (1940). The fish was quickly covered with another glass plate and observed under the microscope. With practice the time elapsing between the initial removal of the flask from the aquarium and the observation of the melanophores did not exceed 6 sec.

When timing colour change from black or white backgrounds to darkness, i.e. taking observations in the dark, a closed box which could be very dimly illuminated from the inside and had a small aperture on the top was covered by a glass plate and used as a support for the grid. At the moment of taking out the flask from the aquarium the box light was switched on so that the position of the fish on the grid could be seen. As soon as the fish was covered the box light was switched off and the fish was examined under a microscope with concealed substage lighting. This extra procedure added about 2 sec. to the time elapsing between removal of the flask and observation of the melanophores.

When timing colour change from darkness to black or white backgrounds the fish were kept in their flasks on trays of the appropriate colour in darkness for 10 days before the light was switched on. The water in the aquarium (and therefore in the flasks) was replaced by means of cautious siphoning every 2 days in order to avoid pollution.

Hogben & Landgrebe (1940) have shown that in the case of *Gasterosteus* records of M.I. values cannot be made from consecutive readings of the same fish, since readings are no longer accurate after the fish has been handled. It therefore seemed advisable to avoid repeated handling of the minnows and to discard a fish after one reading had been taken. Accordingly, each fish was only used once.

Observations of melanophores in unoperated fish were restricted to dermal melanophores in the region already referred to as *B* (§ 1 (*b*)). When a minnow is removed from its flask and observed as described above its melanophores change their form very rapidly, so that it is impossible to make any reasonable estimate of their degree of dispersion in more than one region at a time. Attempts to fix the

melanophores by plunging the fish into hot fixatives did not meet with success (Hogben, 1943; Parker, 1943).

The M.I. readings were plotted graphically, each point on the graph representing the mean M.I. value of a group of at least ten fish. The limits of a vertical line drawn through each point show the lowest and highest M.I. values found within the group.

(*b*) *Observations*

The results presented in this section agree closely with those obtained by Hogben & Landgrebe (1940) with *Gasterosteus* and by Neill (1940) with some other teleosts, and the treatment given by these workers may be applied here.

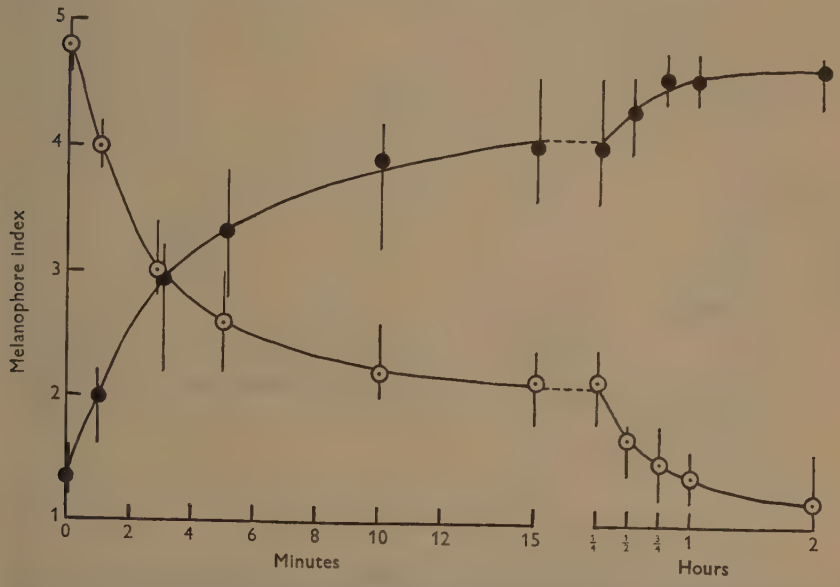


Fig. 3. Responses of the melanophores of region *B* in separate groups of ten unoperated minnows transferred from equilibrium on an illuminated white background to an illuminated black background and vice versa. Temperature: 12 ± 0·2° C. Illumination: 40 W. lamp at 1 m.

In the period between the presentation of a visual background stimulus and the response of the melanophores three components play a part:

(*a*) The time required for the eye to react to the stimulus.

(*b*) The time required for transmission through the co-ordinating system.

(*c*) The reaction time of the melanophores.

(*a*) is only a few seconds. If there is only nervous co-ordination (*b*) is only short and is measurable in seconds. As far as nervous control is concerned (*c*) is also short; e.g. if a decapitated and eviscerated minnow whose melanophores have reached their maximum degree of dispersion is stimulated electrically on the bare surface of its spinal cord the melanophores show a M.I. change from 4·6 to 1·4 in 20–30 sec. After

stopping the stimulus the melanophores return to their original state in little more than 1 min. On the other hand, the melanophores of the minnow only react relatively slowly to hormones, requiring about 2 hr. for a maximum response (Healey, 1940).

Fig. 3 shows the responses of the melanophores when the minnow, fully adapted to an illuminated black background, is transferred to an illuminated white background, and vice versa. There is a rapid colour change for the first few minutes,

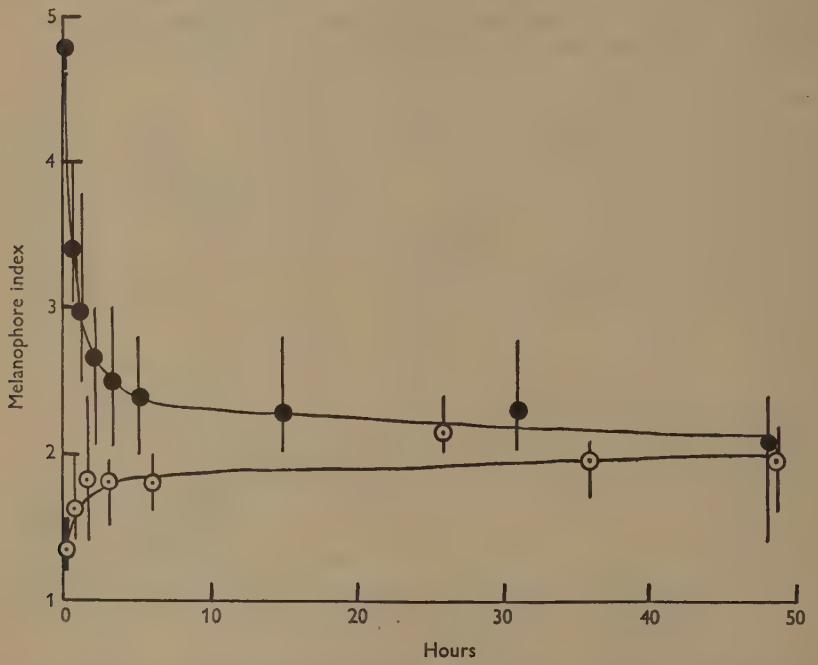


Fig. 4. Responses of the melanophores of region *B* in separate groups of ten unoperated minnows transferred from equilibrium on illuminated black and white backgrounds to darkness. 12 ± 0·2° C., 40 W. at 1 m.

i.e. its rate is comparable with that which we should expect in the case of nervous co-ordination. At the end of 15 min. this rate has become greatly reduced and the colour change continues slowly, only becoming complete after about 2 hr. Other methods (Healey, 1940) have already shown that in the minnow there is not only nervous co-ordination of colour change but also chemical co-ordination through the mediation of at least one hormone. The effects of this double system may be well seen here: the initial rapid colour change is the result of nervous co-ordination, while the slow colour change which follows it is the result of the action of a hormone.

When the animal is fully adapted to an illuminated white background the mean

M.I. value is 1·25. When black-adapted the value is 4·8. Different individuals agree fairly closely.

Fig. 4 shows the responses of the melanophores when the minnow is transferred from equilibrium on black and white illuminated backgrounds to darkness. Equilibrium in darkness is only reached after about 45 hr., a very much longer time than that required for illuminated background equilibrium (Fig. 3). This shows once again (Hogben & Landgrebe, 1940; Neill, 1940) that in teleosts the mechanisms which are responsible for the colour changes following transition from illuminated backgrounds to darkness are different from those following a change of illuminated

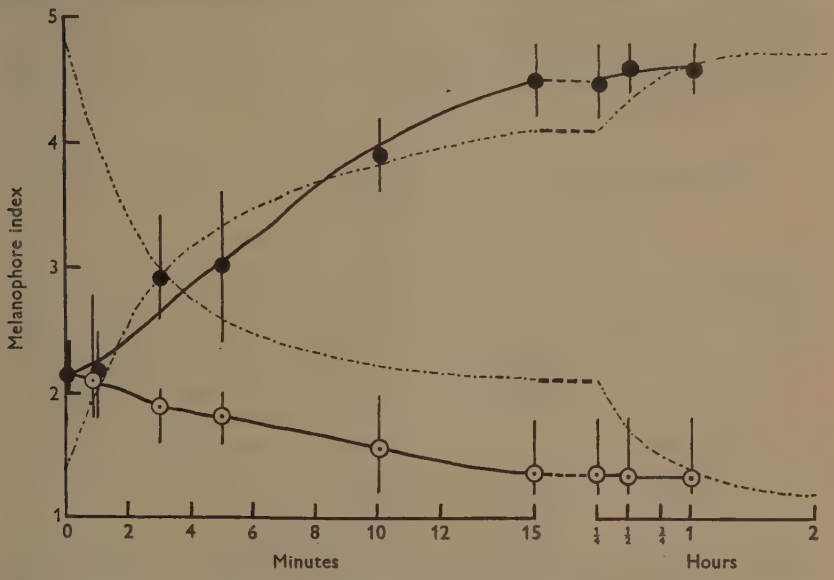


Fig. 5. Responses of the melanophores of region *B* in separate groups of ten unoperated minnows transferred from equilibrium in darkness to illuminated white and black backgrounds. 12±0·2° C., 40 W. at 1 m. Fig. 3 (broken curves) is superimposed for comparison.

background. In the latter case the nervous system controls the rapid colour change and is reinforced by hormonal control; but the slow colour change in darkness is the result of a gradual change in hormone concentration, the nervous system here playing no part.

Fig. 5 shows the responses of the melanophores when the minnow is transferred from equilibrium in darkness to black or white illuminated backgrounds. Here the nervous system again plays a part, and the times are of the same order as those shown in Fig. 3.

The minnow, like many other animals showing colour change (Hogben, 1943), reacts not only to the tint of the background (secondary response) but also to the intensity of illumination (primary response). In some blinded fish observed

macroscopically this primary response is evident within a few seconds of transference from darkness to light; in others it is difficult to detect in this way (Healey, 1940). The minnow when adapted to darkness has a mean M.I. value of 2·1. The blinded minnow under standard illumination has a mean M.I. value of 3·0 after 4 days; but this becomes progressively higher with time, so that it is not possible to use the value for a trustworthy estimate of the extent of the primary response, it can only serve as some indication. The curves presented in this section do not show the presence of this primary response. The reason may lie in the technique adopted together with the fact that the degree of this response varies considerably among different minnows; but probably the technique is the more responsible. The minnow changes colour with extreme rapidity when handled, and even the few seconds required to read the M.I. may be sufficient to render the results suspect as far as absolute values are concerned, while allowing them sufficient accuracy to show the general trends. A different technique may well throw light on this point.

III. THE COLOUR CHANGE OF MINNOWS AFTER SPINAL SECTION BETWEEN VERTEBRAE 5 AND 12

(*a*) *General experimental procedure*

The minnows used in this series of experiments were about 5 cm. long. They were anaesthetized in 0·5% urethane solution and then supported with filter-paper and rubber bands on a piece of grooved paraffin wax in a small tray provided with inlet and outlet tubes. From the former the fish was supplied with a stream of 0·25% urethane which could be temporarily replaced by water if the respiratory movements of the animal became weak. The operation was carried out under a bar binocular with twin lighting, visibility being further assisted by continuous washing of the operation site with a stream of Ringer solution. After sewing up the wound the fish was weighted and suspended with its number ticket in an aquarium painted black or white. The procedure has already been fully described (Healey, 1940). The aquaria were placed in the constant temperature tank and illuminated as already described (§ I(*c*)). At noted intervals the fish were lifted out and examined in a container made of plasticine and glass in such a way that the head of the fish rested in a hollow filled with water, while the tail lay over a glass plate and could be examined through the microscope. The fish usually lay quite still. When working in light the first reading could be made within 6 sec. from the time when the fish was first lifted; in darkness the time required was within 8 sec.

The M.I. was read in the same region (*B*) as in the case of the unoperated fish, but in many cases, since the colour change is now much slower, additional readings were taken in region *A*, in the dorsal surface and in the lateral stripe (§ I(*b*)). In such cases the first position was read again to make sure that the M.I. value had not changed in the time (within 22 sec.). Some results obtained from different regions are presented in § III(*b*). After the M.I. value had been read the fish was replaced in its aquarium.

In making these observations some precautions were taken in an attempt to lessen the danger of subjective influences on the part of the observer. The fish were removed from

their aquaria in different order at different times; they were frequently rearranged in the aquaria; the reference number labels were only looked at after the reading had been taken. As a result the observer had no remembrance of a previous reading of an individual fish and, therefore, expected no particular M.I. value. Such a precaution would hardly have been necessary if all the melanophores in the field of view had shown the same degree of dispersion; but in many cases this was not so, and an estimate had to be made from the general appearance.

It will be noted that in this series of experiments the fish were not discarded after a reading had been taken but were replaced in their aquaria and read repeatedly. In estimating the likelihood of such repeated readings affecting the accuracy of the results the following points deserve consideration:

(i) Unlike the unoperated fish these spinal animals showed very wide variations in M.I. among one another when under the same experimental conditions (cf. figures showing responses of operated and unoperated fish). Consequently, readings derived from different groups of fish gave very irregular graphs whose tendencies could not be well determined. This is a point of some consequence when we bear in mind that the whole object was a determination of time relations as accurately as possible.

(ii) Groups of fish read four times a day gave the same type of curve as did other groups read only once daily. The actual values obtained differed, but such differences could well be attributed merely to the wide variations between individual fish ((i) above).

(iii) Attempts to repeat a series of observations on the same group of fish in a different way (e.g. black to white with frequent observations followed by white to black and then black to white again with infrequent observations) showed that the time relations were of the same order, but that the actual M.I. values were somewhat different as a result of degenerative changes which often appear in the melanophores some time after spinal section.

(iv) Readings were taken of some fish which were then kept for a longer time than usual in the plasticine container. When it was obvious that they had become paler they were returned to their aquarium. Another reading within 2 hr. gave results which agreed well with the other observations, both when a fish was in the process of changing its colour and after it had reached a steady M.I. value.

(v) An experiment was made in which the colour change of a group of fish was read at intervals in the usual way but with this addition: between each reading and not less than 3 hr. before the next reading the fish were lightly pinched with forceps or gently tapped with a glass rod. As a result they became excited and went very much paler (von Frisch, 1911; Smith 1931). In spite of this the readings gave a graph which agreed with the type obtained when no mechanical stimulation was applied.

(vi) One might say that, in any case, the fish had already been most severely 'handled' in the course of the operation and subsequent suspension in aquaria.

In short, unless each observation had been made on such a large group of fish that the individual differences could have been neglected, nothing would have been achieved by using separate groups.

When the observations on a fish were completed the animal was killed by immersing it for 6 sec. in boiling water. After being suspended for 2 weeks in a formol-acetic acid mixture to harden and decalcify it, it was divided by a median vertical longitudinal cut and examined under a low-power microscope. In all the fish included in this paper the site of the spinal section was found to lie between the 5th and 12th vertebrae.

(b) Responses of melanophores from the time of the operation to equilibrium on illuminated black and white backgrounds

The fish taken from the stock tanks had a M.I. of about 3·2. The urethane caused some slight additional darkening, but almost immediately after the cut in the spinal cord had been made the fish became still darker in colour. This darkening continued to increase after the fish had been put on to a black or white background and reached an individual maximum within 30 min. The fish had been back in water for about 25 of these minutes, a time in excess of that required by an unoperated animal to

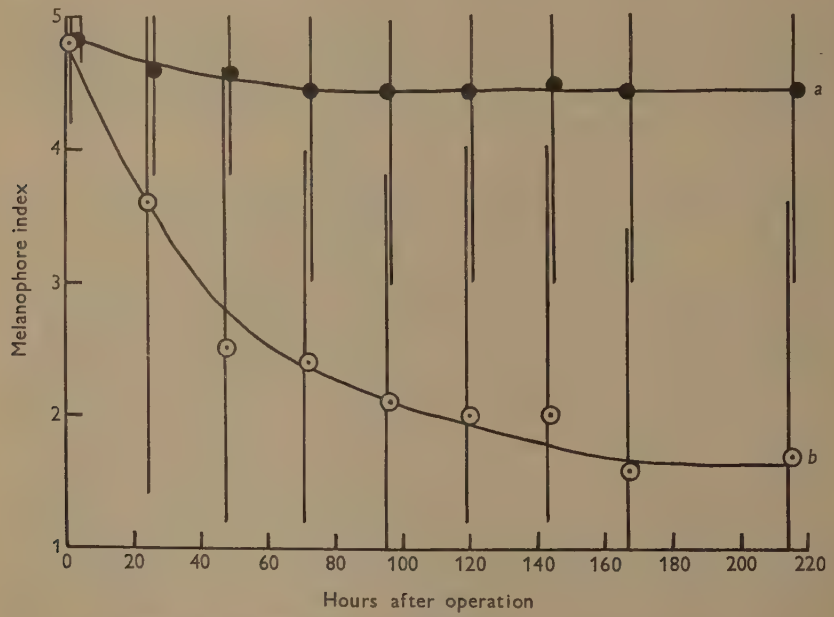


Fig. 6. (*a*) Responses of the melanophores of region *B* in a group of twenty-two minnows from the stock tanks placed on an illuminated black background immediately after spinal section. (*b*) Responses in twelve minnows similarly treated but placed on a white background. 12 ± 0·2° C., 40 W. at 1 m. The first reading in each series was taken about 40 min. after the operation.

recover from the darkening effects of urethane anaesthesia, i.e. the darkening results from the operation. Mention has already been made (§ I(*a*)) of this melanophore dispersion which follows section of chromatic fibres. Further attention will be given to it in § III(*f*).

At noted times after the operation the M.I. values of the fish were read. Fig. 6 shows these readings expressed graphically. Curve (*a*), representing the responses of the melanophores when the fish were placed on an illuminated black background after the operation, shows two things: first, from the mean M.I. values, that the dispersion of the melanophores following the operation becomes less intense and reaches

a steady condition after 4–5 days; secondly, that there is a considerable difference between individuals. Curve (*b*) shows the response when the fish were placed on an illuminated white background after the operation. In this case the M.I. becomes much lower and finally reaches a steady value after 8–9 days. The differences between individual fish on the M.I. scale are very marked. In this connexion it may be emphasized again that the vertical lines in the figures only join the highest and lowest M.I. readings. In fact, for curve *b* (Fig. 6) at 9 days only three fish had M.I. values above the mean, namely, 2·0, 2·6 and 3·6. The other nine fish contributing to this

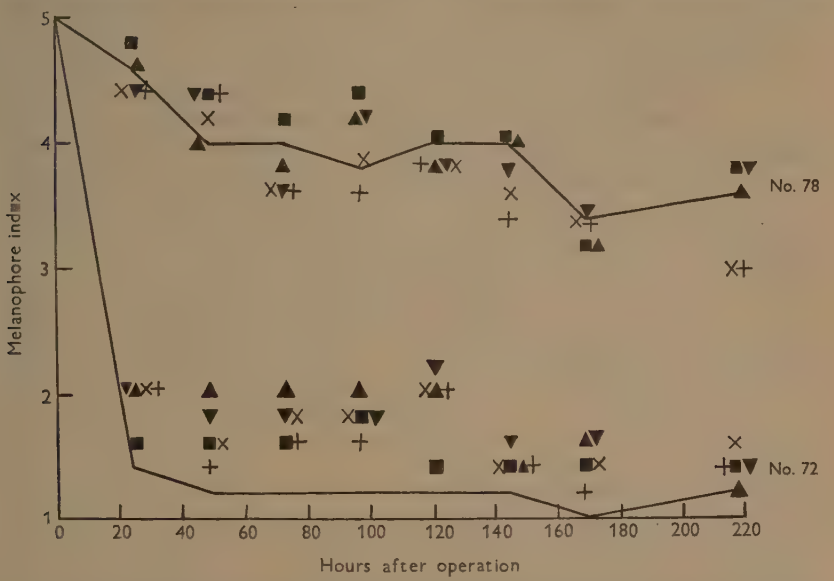


Fig. 7. Responses of the melanophores in different regions of two minnows (nos. 72 and 78) from the stock tanks placed on an illuminated white background immediately after spinal section. 12 ± 0·2° C., 40 W. at 1 m. Continuous line = region *B*; square = *A*; triangle resting on base = lateral stripe epidermis; triangle resting on angle = lateral stripe dermis; × = dorsal surface epidermis; + = dorsal surface dermis. The first reading in each series was taken about 40 min. after the operation.

curve were below the mean, i.e. the chance presence of even one divergent fish in a group can give a long vertical line in this type of representation. Nevertheless, the presence of divergent fish within a group was so common that there was no question of ignoring it and treating such fish as exceptions. Indeed, in most cases the majority of the fish within a group did not conform so nearly to the mean as in the example quoted here.

In the case of the operated fish, as already mentioned, not only was the region *B* (§ I(*b*)) read but often also the region *A* and sometimes the dorsal surface and lateral stripe. Fig. 7 shows the reactions of the melanophores from these different regions in two fish nos. 72 and 78, both of which contributed to Fig. 6. In both cases the plots

of the various melanophore regions in an individual all lie reasonably close together. Since similar results were obtained from other fish, it may be concluded that the plots of region *B* give a fair indication of the behaviour of the melanophores in other regions of these spinal fish. The great difference in the reactions of individual fish has already been pointed out. It is clearly demonstrated in Fig. 7. Fishes nos. 72 and 78 both had a M.I. of 5·0 immediately after the operation, but on an illuminated white background they paled at very different rates and to a very different extent; no. 72 reached a M.I. value of 1–2 in about 30 hr. and subsequently became only a little paler; the M.I. value of no. 78 fell fairly regularly to reach a level of 3–4 after 8–9 days.

(*c*) *Responses of melanophores when spinal fish, equilibrated to an illuminated background, are subjected to background reversal*

Operated minnows which had been allowed to reach a steady M.I. value by keeping them for 9 days after the operation in black and white illuminated aquaria were

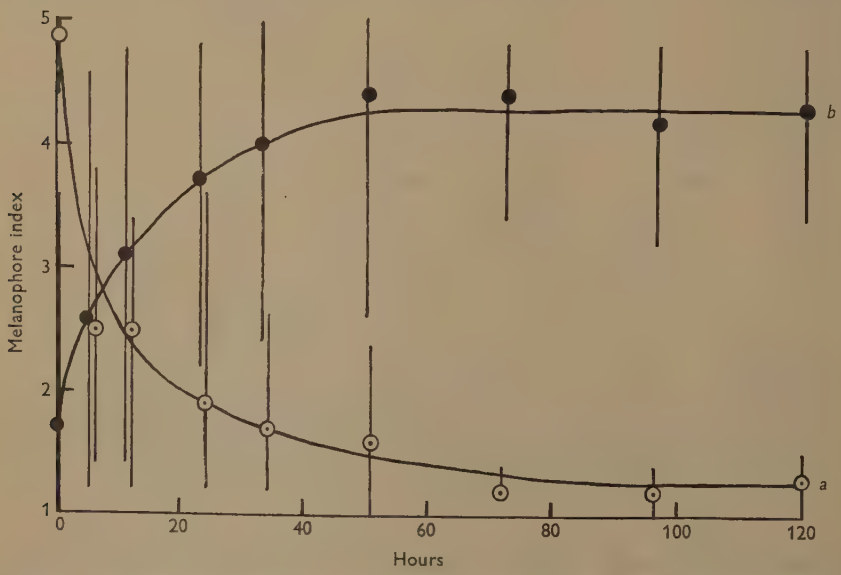


Fig. 8. Responses of the melanophores of region *B* in a group of ten minnows which after spinal section were left for 9 days (*a*) on an illuminated black background and (*b*) on an illuminated white background and then subjected to background reversal (this last colour change being shown in the figure). 12 ± 0·2° C., 40 W. at 1 m.

subjected to a reversal of the background to which they had become equilibrated. Fig. 8 shows the results of such an experiment. Considering first the background change from black to white, shown in curve (*a*): the group of fish had reached a mean M.I. value of 4·9 after the preliminary period of 9 days on a black background

after the operation. In Fig. 6 the group of fish left on a black background showed a lower M.I. value after the same time. But it has been pointed out that there is considerable variation; in the present group (Fig. 8) the mean remained high. After placing the fish on a white background this initial M.I. value fell to 3·0 in 5 hr., to 1·5 in 48 hr. and to 1·25 after another 48 hr. The curve 8(*a*) also shows that the later M.I. values are all much closer together than they are in the case of the white-equilibrated fish in Fig. 6(*b*). This would appear to be nothing more than a matter of chance; the final variation in this group happened to be slight.

Considering, secondly, the background change from white to black, shown in curve 8(*b*): the group of fish had reached a mean M.I. value of 1·7 after the preliminary period of 9 days on a white background. When the group was placed on a black background the mean M.I. value rose to 2·6 in 5 hr. and to 4·25 after about 50 hr. After this it remained at a steady level.

The two curves in Fig. 8 show time relations of a different order from those obtained from unoperated minnows (Fig. 3). They resemble the time curves associated with amphibian colour change (Hogben & Slome, 1931). The reaction time to pituitary hormones of the melanophores of the minnow under these conditions of operation is of the order of 90 min. (Healey, 1940) and the eye is still the receptor. The long time required for colour change is therefore consistent with the hormonal control existing in this fish.

The final M.I. values indicated by curves 8(*a*) and (*b*) are not the same as the initial values of 8(*b*) and (*a*). This might be attributed to the differences which have already been seen to exist between individuals.

(*d*) *Responses of melanophores in operated minnows equilibrated to illuminated black and white backgrounds on transference from light to darkness and the reverse*

The primary response is more obvious in the operated than in the normal minnow, since in the former it is not rapidly masked by interference from the nervous system resulting from visual background stimuli. The operated minnow equilibrated in darkness has a M.I. value around 3·6. (Again there is considerable variation.) The blinded spinal minnow in light gradually becomes darker, the depth of colour increasing for many days, so that it gives us no reliable information about the extent of the primary response. However, the spinal but seeing fish equilibrated to darkness shows the primary response quite clearly when it is exposed to light; the change in mean M.I. value is from about 3·6 to about 3·8 in Fig. 10. Similarly, the spinal illuminated fish shows a relatively rapid initial paling when transferred from light to darkness; from about 4·6 to 4·2 and 2·4 to 1·9 in Fig. 9. This response requires about 6 min. for its approximate completion. It appears to be largely, if not entirely, the result of local stimulation of the melanophores (Healey, 1940).

Fig. 9 shows the responses of the melanophores of spinal minnows which have been equilibrated to an illuminated black or white background and then transferred to darkness. The fish from a black background show a fairly rapid fall in the M.I. value as a result of the primary response. Thereafter the fall continues slowly and

reaches a steady value of 3·6 after about 100 hr. The fish from a white background, after showing the initial fall in M.I., gradually become darker, reaching a steady value after about 160 hr. Here, as in the similar case in § II(*b*), the hormone secretion which supplements rapid nervous action in the intact minnow is no longer taking place through stimulation of the eyes. Its slow change in concentration results in the new equilibrium level for darkness.

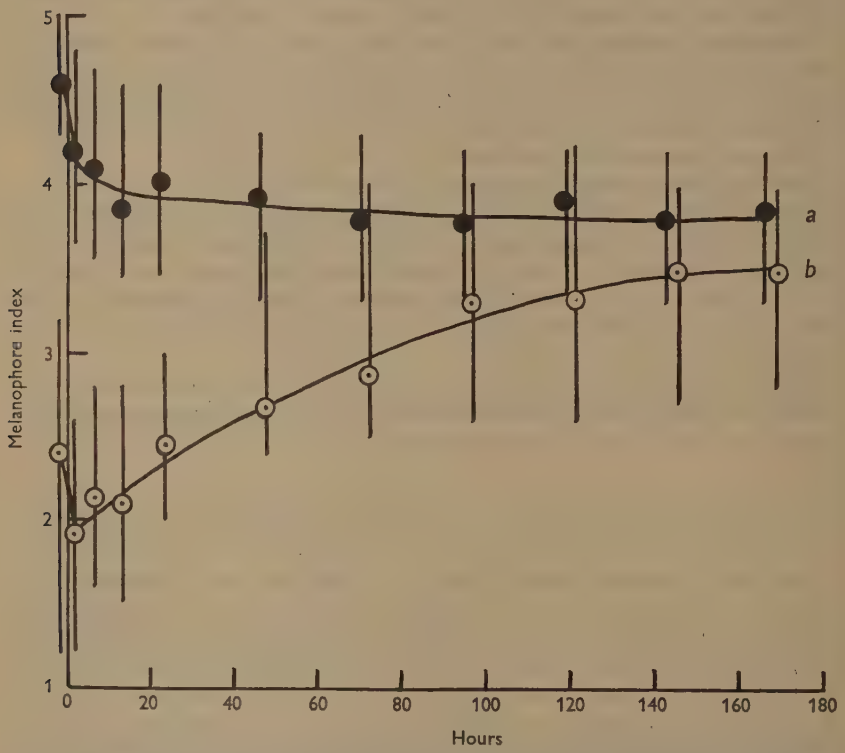


Fig. 9. Responses of the melanophores of region *B* in a group of ten minnows which after spinal section were equilibrated to an illuminated black or white background and then transferred to darkness. 12±0·2° C., 40 W. at 1 m.

When such fish, equilibrated to darkness, are transferred to illuminated black and white backgrounds, the establishment of new levels of hormone concentration in response to the optic stimuli results in the attainment of new equilibria, as shown in Fig. 10. These are not the same fish as those used for Fig. 9, the initial values of the two groups being here quite close to one another (M.I. 3·6 and 3·7). Very soon after the light is switched on the primary response is seen and the mean M.I. rises (to 3·8 and 3·9). The fish on a black background then darken slowly to reach a steady value

after about 120 hr. The fish on a white background become paler and reach a steady value in about the same time.

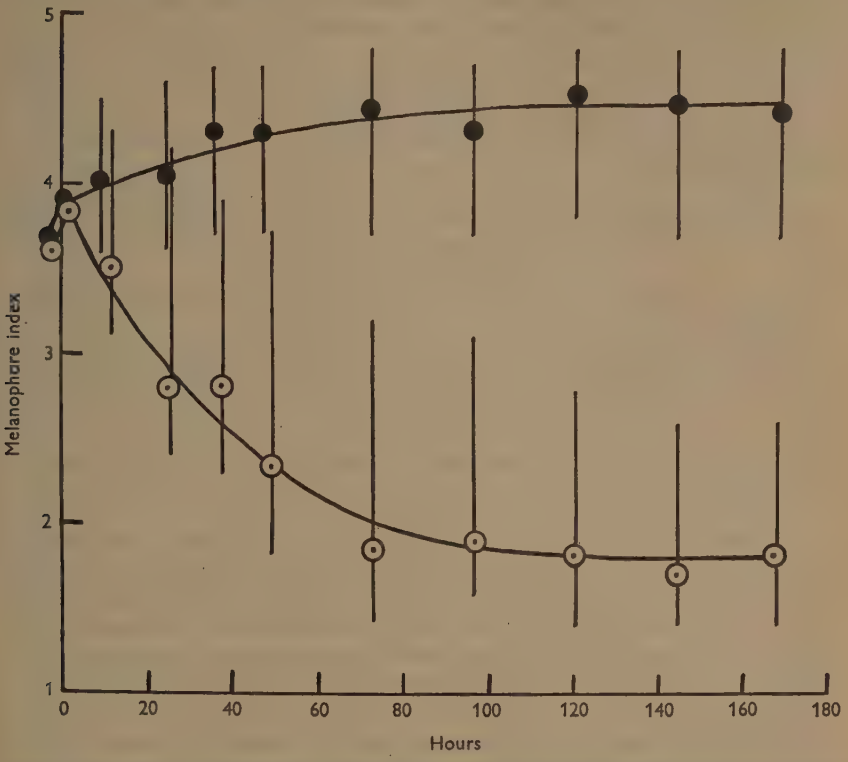


Fig. 10. Responses of the melanophores of region *B* in a group of ten minnows which were successively subjected to spinal section, 9 days on an illuminated black (or white) background, 8 days in darkness and an illuminated black (or white) background (this last colour change being shown in the figure). 12±0·2° C., 40 W. at 1 m.

(*e*) *A consideration of the responses of the melanophores with regard to the bi-humoral hypothesis*

These results obtained with spinal minnows increase the existing knowledge of hormonal control in this fish (Healey, 1940) and agree with results obtained with other teleosts by Hogben and his co-workers (Hogben, 1943). According to Hogben's hypothesis (i) the colour changes resulting from the transition from light to darkness and vice versa are the result of excretion or secretion of the *B* (melanophore dispersing) hormone from the *pars intermedia* of the pituitary gland; and (ii) those following transference from black to white illuminated backgrounds and vice versa are the result of secretion or excretion of *W* (melanophore aggregating) hormone or of direct nervous control '*secondarily* superimposed upon, and to a greater or less extent

replacing, a more archaic humoral mechanism' (Hogben & Landgrebe, 1940). This hypothesis is supported by the results obtained from the timing of colour changes in Amphibia (Hogben & Slome, 1936), elasmobranchs (Waring, 1938) and the eel (Neill, 1940), the relevant times being those required for the change

(*a*) from equilibrium on a white background in light to equilibrium on a black background in light

$({}_{w}T_{b}) = 60$ hr. (Fig. 8);

(*b*) from equilibrium on a black background in light to equilibrium on a white background in light

$({}_{b}T_{w}) = 90$ hr. (Fig. 8);

(*c*) from equilibrium on a white background in light to equilibrium in total darkness

$({}_{w}T_{d}) = 160$ hr. (Fig. 9);

(*d*) from equilibrium on a black background in light to equilibrium in total darkness

$({}_{b}T_{d}) = 100$ hr. (Fig. 9);

(*e*) from equilibrium in total darkness to equilibrium on a white background in light

$({}_{d}T_{w}) = 120$ hr. (Fig. 10);

(*f*) from equilibrium in total darkness to equilibrium on a black background in light

$({}_{d}T_{b}) = 120$ hr. (Fig. 10).

The presence of a *W* hormone causing melanophore aggregation in the minnow has already been demonstrated (Healey, 1940), but the existence of a hormone which causes melanophore dispersion in this fish, although strongly indicated, needs further confirmation. The colour changes of the spinal fish which have been described in this section do not indicate that any active nervous response to background tint is playing a part in them. Nevertheless, the work of von Gelei (1942) suggests that dispersing fibres which have not been cut by the spinal operation may still be influencing the melanophores: that is, there exists the possibility, not precluded by the evidence at hand, that these fibres may at least be responsible for some slight melanophore dispersing influence which is either (*a*) constant under all conditions of illumination or (*b*) greatest in light and least in darkness. These possibilities require further investigation but do not concern the present argument. The order of time required for the colour changes under consideration can only be associated with hormonal control. Following Hogben and his co-workers we may argue thus: If there is only the *W* hormone playing a part its presence in varying amounts in the blood will be responsible for the colour changes observed, namely, pale on an illuminated white background (high concentration of *W*), intermediate in darkness (medium concentration of *W*), dark on an illuminated black background (low concentration of *W*). Therefore transition from equilibrium on a black background to equilibrium on a white background should involve the liberation of more *W* hormone than transition from a black background to darkness or the transition from darkness to a white background, i.e. ${}_{b}T_{w}$ should be greater than ${}_{b}T_{d}$ and ${}_{d}T_{w}$. But the figures given above show that this is not so. Again, the transition from equili-

brium on a white background to equilibrium on a black background should involve the elimination of more *W* hormone than transition from a white background to darkness or the transition from darkness to a black background, i.e. ${}_wT_b$ should be greater than ${}_wT_d$ and ${}_dT_b$. Again the figures show that this is not the case. By this argument, therefore, the colour changes observed in the operated minnow cannot be due to a single *W* hormone. We therefore have a further indication of the presence of another hormone, *B*, antagonistic to the *W* hormone and causing melanophore dispersion in the minnow. The different rates of accumulation and elimination of these two competing hormones can then be made to account for the various times elapsing between establishment of different equilibrium conditions.

(*f*) *A consideration of the responses of the melanophores with regard to their double innervation*

In § I(*a*) reference has been made to some observations which indicate that melanophores are supplied with both aggregating and dispersing nerve fibres. Parker and his school maintain that the cutting of chromatic nerve fibres causes darkening through stimulation of dispersing fibres, i.e. the darkening which follows the operation of spinal section, as described in the present paper, might be interpreted as being a result of such stimulation. On the other hand, according to von Gelei (1942), dispersing fibres in the minnow pass out of the spinal cord well before the level of the cuts made in this series of experiments. There is thus lack of agreement on this point, and further work is necessary to clarify it. However, some of the results obtained here do merit mention in this connexion.

(i) A comparison of Figs. 6(*b*) and 8(*a*) shows that, although the initial M.I. values are about the same in both, the rate of paling is much greater in the latter. Protagonists of the theory that cutting stimulates dispersing fibres might say that this is another example to prove their point. They might perhaps add that von Gelei's description of the track of the dispersing fibres cannot be complete; that there are, in addition, other dispersing fibres which follow the same path as those causing aggregation and are therefore cut by the present spinal section; that after some time the stimulating effect of the cut decreases and the melanophores are now affected only by the hormonal constitution of the blood and by von Gelei's dispersing fibres which, according to him, do not seem to play a very active part. Antagonists of the theory of stimulation through cutting might say, as readily, that the paralysis of aggregating fibres is not the only result of spinal section. The severing of their nervous connexions with the brain may cause considerable physiological changes in the melanophores which may well affect their reactivity to hormones; also, spinal section is, at the best, a violent and undiscriminating operation.

(ii) It is further of interest in this connexion to compare the rates of paling on an illuminated white background of spinal fish which have received different preliminary treatment (Healey, 1940). The fish represented in Fig. 6 were kept in the stock tanks before their spinal cords were cut. The curves shown in Fig. 11 were obtained thus: two groups of unoperated fish, (*a*) and (*b*), were placed respectively on black and white illuminated backgrounds. At the same time another group (*c*) was subjected

to spinal section and placed on a black background. After 9 days groups (*a*) and (*b*) were also operated and all three groups were then placed together on a white background. Their M.I. values were then read and plotted to give Fig. 11. In this experiment it was not convenient to use the thermostatically controlled tank, and the temperature accordingly varied between 13·5 and 15·0° C.; but this variation applied equally to all three groups so that the results are comparable with one another

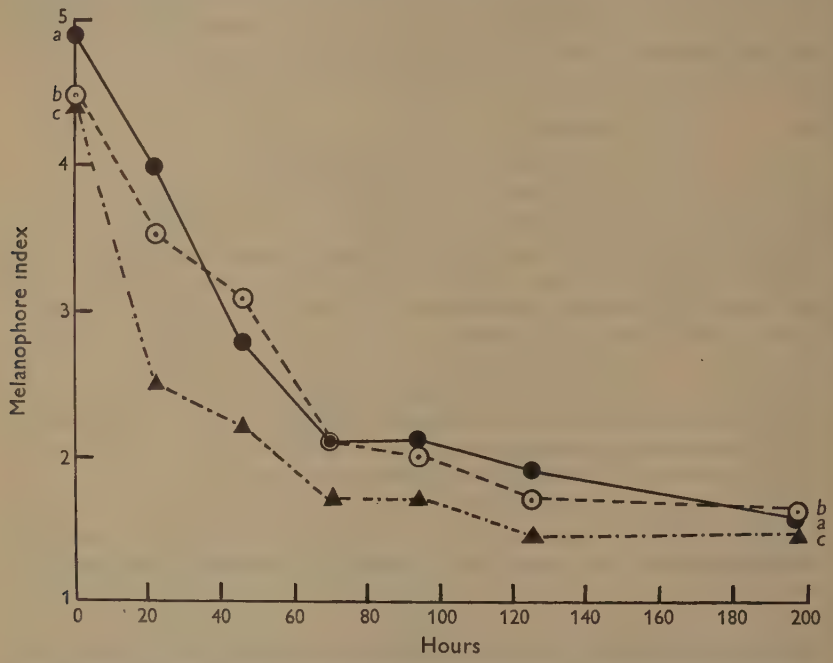


Fig. 11. Responses of the melanophores of region *B* in three groups of ten minnows treated as follows: (*a*) subjected to spinal section after 9 days on an illuminated black background and then placed on an illuminated white background; (*b*) subjected to spinal section after 9 days on an illuminated white background and then replaced on an illuminated white background; (*c*) subjected to spinal section, placed on an illuminated black background for 9 days and then on an illuminated white background. 13·5–15·0° C. for all fish simultaneously; 40 W. at 1 m. The first reading in each case was taken about 40 min. after the operation.

if not with Figs. 6 and 8 (12° C.). We see that in groups (*a*) and (*b*) the previous adaptation to black and white backgrounds made no difference to the effect of spinal section excepting that group (*a*) was rather darker than group (*b*) immediately after the operation and for the next 30 hr. This first difference in colour may be attributed to the very different concentrations of hormones in the blood of the two groups at the time of the operation. After 30 hr. on a white background this difference had become much reduced. Otherwise, in spite of the previous black and white adaptation, there was no essential difference between the rates of paling of the two groups.

Group (*c*) became paler more quickly than did groups (*a*) and (*b*), a result which agrees with the comparison already made above between Figs. 6(*b*) and 8(*a*). One may draw the conclusion that this first paling immediately after the operation is a result of processes which are not identical with those responsible for paling which may occur later, i.e. the operation of cutting the spinal cord would appear to initiate some process which, for a time, actively combats the paling of the fish.

(iii) The slow changes in hormone concentration which result when the unoperated minnow is transferred from illuminated black or white backgrounds to darkness are seen in Fig. 4; the M.I. value at equilibrium in darkness is about 2·1. Fig. 9 shows the chromatic behaviour of the spinal minnow under similar conditions; here the M.I. reaches a steady value of about 3·6. Further, the condition of darkness equilibrium in the case of the spinal minnow is only reached after a much longer time than that required by the unoperated minnow. These differences in behaviour of normal and spinal minnows might possibly be claimed as evidence for double innervation; thus the suggestion might be made that in the unoperated fish in darkness the parts of the nervous system responsible for aggregation and dispersion of the melanophores might be competing weakly for control with the former dominant. Then, although the hormone concentrations at the end of 45 hr. are not in darkness equilibrium, the nerves alone might be responsible for the steady state of the melanophores as shown by the present technique. Secondly, in the spinal fish only the dispersing system indicated by von Gelei is playing an active part (at least, so far as connexions with centres in the brain are concerned). Acting against this, in the case of the spinal fish transferred from equilibrium on an illuminated white background to darkness, is a considerable concentration of *W* hormone. As the latter is gradually eliminated the M.I. value rises but takes longer to reach equilibrium, as it now has to pass through a greater range. Similar arguments might be advanced to account for the chromatic behaviour of the spinal fish transferred from an illuminated black background to darkness. On the other hand, those who are not convinced that dispersing fibres play an active part might say that aggregating fibres maintain a certain slight tonic effect and produce a darkness equilibrium at a fairly low M.I. value in the unoperated fish; in the operated fish, transferred from an illuminated white background to darkness, this effect is no longer exerted, and the *W* hormone concentration has to fall much further so that there is a longer time required to reach the steady state. Finally, one might express the view that the nervous system may play no part in darkness equilibrium in either the unoperated or the operated fish, but that cutting the spinal cord may produce physiological changes in the melanophores themselves which profoundly affect their reactions. In short, the interpretation of these results is uncertain and further experimental work is necessary.

SUMMARY

1. Records were made of the times required for the melanophores of the normal minnow to reach equilibrium when the fish is transferred from one to another of the following conditions: on an illuminated white background; on an illuminated black background; in darkness.

2. These times give further evidence of the parts played by nervous and hormonal mechanisms in the colour change of the minnow.

3. After section of the spinal cord between the 5th and the 12th vertebrae the fish darkens but gradually becomes pale again if kept on an illuminated white background.

4. Such fish can still show a slow colour change: dark on a black background, pale on a white background and intermediate in darkness.

5. Observations of the times required for these colour changes in the spinal minnow show that these no longer resemble those associated with the unoperated fish; rather, they resemble the time intervals associated with amphibian colour change.

6. Further consideration of the times required for colour change in the spinal minnow indicate that there is not only a hormone causing aggregation of the melanophores but also a hormone causing melanophore dispersion.

7. The part played by double innervation of the melanophores is considered.

The work described here was carried out in the Department of Natural History, Aberdeen University, with the help of a grant from the Rockefeller Foundation. This is gratefully acknowledged. The author's thanks are due to Prof. Lancelot Hogben for administering this grant for his benefit and also to Mr R. M. Neill for his readiness to provide facilities in Prof. Hogben's absence. Further experiments were carried out at Aberystwyth. These were made possible by the facilities provided by Prof. T. A. Stephenson and to him also the author wishes to record his thanks.

REFERENCES

Abramowitz, A. A. (1936). Physiology of the melanophore system in the catfish, *Ameiurus*. *Biol. Bull. Woods Hole*, **71**, 259–81.

Abramowitz, A. A. (1937). The role of the hypophysial melanophore hormone in the chromatic physiology of *Fundulus*. *Biol. Bull. Woods Hole*, **73**, 134–42.

von Frisch, K. (1911). Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Pflüg. Arch. ges. Physiol.* **138**, 319–87.

von Gelei, G. (1942). Zur Frage der Doppelinnervation der Chromatophoren. *Z. vergl. Physiol.* **29**, 532–40.

Giersberg, H. (1931). Der Farbwechsel der Fische. *Z. vergl. Physiol.* **13**, 258–79.

Healey, E. G. (1940). Über den Farbwechsel der Elritze (*Phoxinus laevis* Ag.). *Z. vergl. Physiol.* **27**, 545–86.

Hogben, L. T. (1936). The pigmentary effector system. VII. The chromatic function in elasmobranch fishes. *Proc. Roy. Soc.* B, **120**, 142–58.

Hogben, L. (1943). Croonian Lecture: Chromatic Behaviour. *Proc. Roy. Soc.* B, **131**, 111–136.

Hogben, L. & Landgrebe, F. (1940). The pigmentary effector system. IX. The receptor fields of the teleostean visual response. *Proc. Roy. Soc.* B, **128**, 317–42.

Hogben, L. & Slome, D. (1931). The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc.* B, **108**, 10–53.

Hogben, L. & Slome, D. (1936). The pigmentary effector system. VIII. The dual receptive mechanism of the amphibian background response. *Proc. Roy. Soc.* B, **120**, 158–73.

Lundstrom, H. M. & Bard, P. (1932). Hypophysial control of cutaneous pigmentation in an elasmobranch fish. *Biol. Bull. Woods Hole*, **62**, 1–9.

Neill, R. M. (1940). On the existence of two types of chromatic behaviour in teleostean fishes. *J. Exp. Biol.* **17**, 74–98.

Osborn, C. M. (1938). The role of the melanophore-dispersing principle of the pituitary in the color change of the catfish. *J. Exp. Zool.* **79**, 309–30.

Parker, G. H. (1934). Color changes of the catfish *Ameiurus* in relation to neurohumors. *J. Exp. Zool.* **69**, 199–223.

PARKER, G. H. (1938). Melanophore responses in the young of *Mustelus canis*. *Proc. Amer. Acad. Arts Sci.* **72**, 269–82.

PARKER, G. H. (1943). Methods of estimating the effects of melanophore changes on animal coloration. *Biol. Bull. Woods Hole*, **84**, 273–84.

PARKER, G. H. (1948). *Animal Colour Changes and their Neurohumours*. Cambridge University Press.

POUCHET, G. (1876). Des changements de coloration sous l'influence des nerfs. *J. Anat. Physiol.* **12**, 1–90, 113–65.

SMITH, D. C. (1931). The influence of humoral factors upon the melanophores of fishes, especially *Phoxinus*. *Z. vergl. Physiol.* **15**, 613–36.

VILTER, V. (1941). La livrée pigmentaire de l'Anguille et sa régulation physiologique. 11. Physiologie de la livrée xantho-mélanique dans ses rapports avec la morphogenèse pigmentaire. *Bull. Histol. Tech. micr.* **18**, 145–66.

WARING, H. (1938). Chromatic behaviour of elasmobranchs. *Proc. Roy. Soc.* B, **125**, 264–82.

THE DISTRIBUTION OF ELECTROMOTIVE FORCES IN THE NEIGHBOURHOOD OF APICAL MERISTEMS

By A. L. McAULAY, J. M. FORD and A. B. HOPE
Physics Department, University of Tasmania, Hobart, Tasmania

(*Received 21 December* 1950)

(With Four Text-figures)

I. INTRODUCTION

The experiments to be described form part of the early stages of a research whose general object is to investigate the organization of plant material as it develops from a meristematic mass to differentiated tissue. Two major classes of control can be recognized in the organization of any living material, one, characteristic of and located in the cells of the developing organs and the other characteristic of the surroundings of the changing cells. This second controlling factor has been named the biological field, and much is known of its properties in the case of animal embryology.

It is a striking fact that wherever a biological field can be recognized and the electrical condition of the region tested, an electrical field is found to be present. An investigation of the behaviour of the electrical field while organization of plant tissues is in progress appears therefore to offer a promising lead to knowledge of the biological field.

Early experiments made in this laboratory and a study of the literature showed a surprisingly erratic behaviour of the electric potentials measured in plant tissues. For this reason attention was given to the question of explaining this and finding a satisfactorily reproducible background for the description of the normal electrical condition of a growing plant.

An examination of the literature reveals that most investigators are in agreement in finding the regions adjacent to apical meristems electrically positive to older parts of the organs, *on the surface of the plants*. Such a polarity has been found by Thomas (1939), using bean (*Vicia Faba*) roots, Ramshorn (1934), using sunflower hypocotyls and asparagus shoots, and Lund (1947), working with onion roots. However, some of the results of Clark (1937) are contrary to these findings. He states that the apices of *Pisum*, *Vicia* and *Impatiens* seedlings are negative to their bases. As the plants mentioned have apical meristems, it is seen that Clark's findings are contrary to those of the aforementioned authors, though his results on the external polarity of *Avena* and *Zea* coleoptiles agree with those of Lund. The results of Lundegårdh (1940) are on the whole not inconsistent with the results of the first three authors, if attention is limited to the first 8–10 mm. from the apex.

The following experiments were carried out to determine the distribution of e.m.f.'s both inside and outside the plant tissue in the neighbourhood of the apical meristems in turnip (*Brassica Rapa*) seedling hypocotyls and in maize (*Zea Mays*) seedling roots. The main results have been checked by experiments with other plants.

Particular attention was paid in this work to obtain unambiguous experimental results under conditions of specified control so that a sound background might be developed for further work.

It is shown that the measured potential is due to electromotive forces produced by the plant and not to static charges on its surface. The plants studied supplied power, through external conductors, of the order of 10^{-9} W. from approximately 2 mm.^{3} of tissue without change in the e.m.f. generated by the plants.

The e.m.f.'s in the neighbourhood of apical meristems were obtained by slightly different techniques each designed to check the other. In all of these the measurements gave (i) potential differences on the surface of the plants between the meristematic region and locations away from it and (ii) potentials between the inside and surface of the plants in the region of the meristem and the corresponding p.d.'s a few millimetres away from the meristem. The results in all cases showed a greater p.d. in the meristematic region than in the region remote from it, and it is thought that a firm foundation is laid for the theory that the meristematic mass is negative to the differentiating tissue that derives from it.

It is recognized that the individual radial p.d.'s measured are functions of external factors such as concentration of ions in the contracting medium, but it is considered that the experiments with their varied techniques establish the reality of the difference between the radial p.d.'s in different regions. It is important to recognize, however, that when other meristems are near an apex, as, for example, when secondary roots develop from a primary root in bean, the essential nature of the potential pattern may be obscured.

II. APPARATUS

The voltage measurements were made with a high input resistance, three-valve electrometer using a type 954 pentode connected to give a grid current of the order of 10^{-14} amp. (Nielsen, 1947) and followed by two stages of direct-coupled amplification. The plant voltage was applied between the isolated, highly insulated grid and an earth connexion, and the output read on a 0–50 μA. meter. The maximum sensitivity was 2·5 μA./mV. A voltage-regulated power supply was used, and zero drift reduced to that due to discharging the filament accumulators.

Electrical contact with the plant to be measured was made with two non-polarizable Ag/AgCl electrodes which consisted of glass tubes containing agar jelly saturated with AgCl into which dipped an AgCl-coated Ag wire. A copper wire soldered to this was connected to the electrometer. The electrodes were made in a photographic dark-room and painted black to reduce the rate of reduction of the AgCl.

The electrodes were placed in the ends of glass tubes with pointed tips containing agar jelly and 0·3 % Knop's solution. In making measurements on the surface of the plants small agar-Knop's solution blobs were placed on the appropriate spots on the plant and the glass tips rested on these. The glass tips were of the order of 20 μ diameter, which is less than the length of most of the plant cells encountered in the experiments. The small p.d. existing between parts of the electrode system alone was subtracted from each reading.

III. MATERIAL

Turnip hypocotyls and maize seedling roots were used as the main experimental material, a few of the experiments being repeated with onion roots, turnip secondary roots, oat roots, and bean seedling hypocotyls. The seeds were germinated on wet filter-paper in Petri dishes in an incubator at a temperature of about 30° C. The hypocotyl of the 2–4-day turnip seedling ranges in length from approximately 3 mm. in a 2-day plant to approximately 20 mm. in a 4-day plant. The meristematic region is at the top of the hypocotyl immediately below the cotyledons. The vascular tissue is like that in the root and is confined to the centre of the hypocotyl. Cells at the top of the hypocotyl are packed closely together and are about 40 μ in length. At the base of the hypocotyl the cells range between 100 and 200 μ in length. The average diameter of the hypocotyl at the meristem is 0·3 mm. and nearer the base is 0·7 mm.

The maize roots used were from 20 to 100 mm. long with a diameter of 1–1·5 mm. The diameter of the stele alone is rather less than half of this. The meristematic region is in the first 1 or 2 mm. of the root tip.

The seedlings were transferred from the Petri dishes to a paraffin-wax insulated glass dish, and the hypocotyl or root rested on paraffin wax during subsequent measurement. The seedlings were disturbed as little as possible during the transfer and were allowed to assume a relatively stable potential before measurement. This usually took 3–5 min. While under experiment they were grown in a moist atmosphere with the root supplied with water from wet cotton-wool. The cell containing the plant was placed on a stage and observed with a dissecting microscope. The illumination was from a 24 W. spot-lamp. The contact tips described above were moved by means of Fonbrune pneumatic micromanipulators.

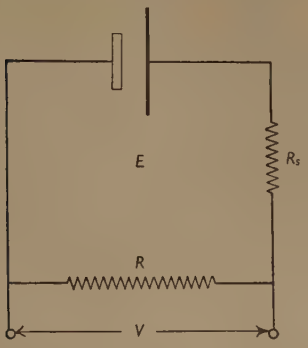
IV. THE PLANT AS A GENERATOR OF POWER

If the plant is an ohmic system, it can be represented by the circuit of Fig. 1 in which E is the equivalent electromotive force and R_s the plant's equivalent resistance. Experiments such as the one to be described have shown that this is so, and that there is no polarization of the e.m.f., that is, E remains constant with currents of the order of 10^{-8} amp. flowing in the circuit. The circuit is completed from two spots on the surface of the plant through different resistances, the potential drop across these being measured with an electrometer of practically infinite resistance.

Referring to Fig. 1, if E is constant, and if R_s is constant and obeys Ohm's law, it may be shown that the following relation exists between V, the p.d. measured on the electrometer, and R, the external shunting resistance:

$$\frac{1}{V}=\frac{1}{E}+\frac{R_s}{E}\left(\frac{1}{R}\right),$$

that is, a linear relation connects $1/V$ and $1/R$. Such a relation has been obtained in experiments on both couch grass (*Agropyrum repens*) shoots and turnip hypocotyls. Fig. 2 shows a graph of $1/V$ plotted against $1/R$ for two spots on the surface of a 3-day-old turnip seedling. From the slope of such graphs and the Y-axis intercepts



$$\frac{1}{V} = \frac{1}{E} + \frac{R_s}{E}\left(\frac{1}{R}\right)$$

Fig. 1. Diagram showing the equivalent circuit of a plant. E is the equivalent electromotive force R_s the equivalent resistance, V is the measured p.d. and R the external resistance.

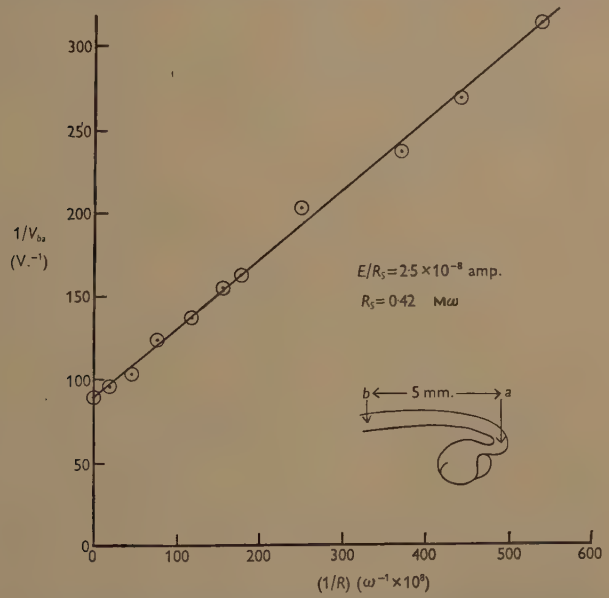


Fig. 2. Graph of $1/V_{ba}$ (in reciprocal volts) against $1/R$ (in reciprocal ohms $\times 10^8$) for two positions a and b on the surface of a 3-day-old turnip seedling hypocotyl.

can be found the values of E and R_s. (R_s calculated from the graphs agreed reasonably well with direct readings obtained with a valve ohmmeter.)

It is concluded that no polarization effects are set up in the plant for the range of currents considered. That is, E remains constant in value even though the plant is nearly short-circuited externally and is supplying current of the order of 10^{-8} amp. (see Fig. 2). This maximum current averaged 3×10^{-8} amp. for the turnip and 6×10^{-8} amp. for couch grass. This corresponds to an average power generation of 10^{-9} and $2{\cdot}5 \times 10^{-9}$ W. for turnip and couch grass respectively.

The threshold value of the current required to polarize the plant e.m.f. is not yet known but is suspected to be of the order of 10^{-7} amp. (Compare Lund and Berry's (1947) value of about 4×10^{-8} amp. for the onion root.)

V. THE DISTRIBUTION OF E.M.F.'s IN THE NEIGHBOURHOOD OF APICAL MERISTEMS. THE SURFACE-POTENTIAL GRADIENT

Numerous experiments have shown that there exists a potential gradient on the surface of 3–5-day-old maize roots and turnip hypocotyls, the region near the meristem being nearly always positive to the older parts of the organ. In older plants, activity developing farther back in roots may cause these regions to become

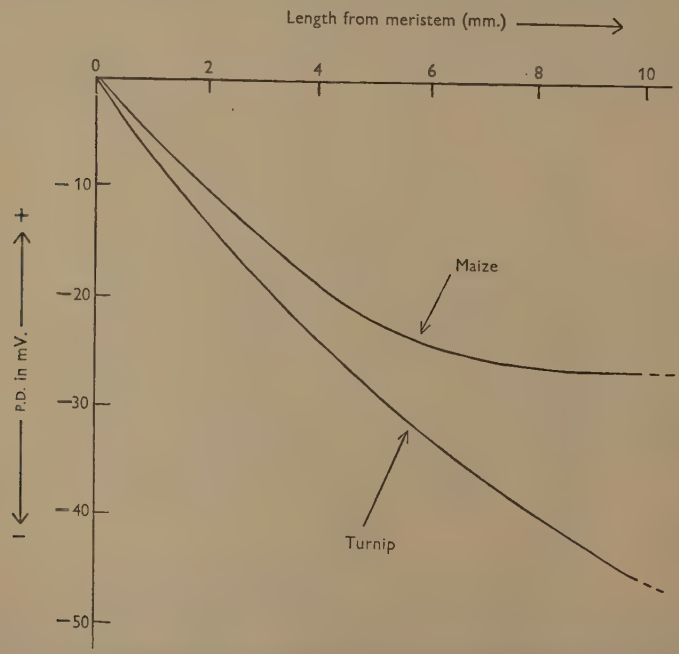


Fig. 3. Graphs of average potential in millivolts against distance from meristematic region in millimetres for twenty-five maize roots and thirty-four turnip hypocotyls.

more positive than the primary apex. The potential decreases approximately linearly with length over the first 6–8 mm. from the meristem.

Fig. 4 shows the average potential gradients. For the turnip hypocotyl, this average is

$$*V_{ab}/l_{ab} = -7{\cdot}1 \pm 3{\cdot}8 \text{ mV./mm.} \quad \text{(from 34 experiments)},$$

where a represents a point on the surface near the meristematic region and b a point on the surface removed from it. l_{ab} is the length between the points a and b. For the growing portion of the maize root this figure is

$$V_{ab}/l_{ab} = -5{\cdot}7 \pm 2{\cdot}4 \text{ mV./mm.} \quad \text{(from 25 experiments)}.$$

It will be shown in the next section that this surface p.d. can be thought of as the sum of two radial e.m.f.'s and a small longitudinal p.d.

VI. THE DISTRIBUTION OF E.M.F.'s IN THE NEIGHBOURHOOD OF APICAL MERISTEMS. THE POTENTIAL DIFFERENCES BETWEEN THE SURFACE AND CENTRE OF THE PLANT

(1) *Results*

When measurements are attempted to obtain p.d.'s inside plant organs, considerable trouble is experienced in getting constant results. This variability is due, at any rate in part, to permanent injury and temporary stimulation. The effects of damage by exploring tips is more marked in turnip hypocotyls than in maize roots. In all cases, however, when appropriate precautions were taken, the results could be represented by the diagram shown in Fig. 4A, which should be examined together with Fig. 4B, a diagrammatic representation of the plant showing where measurements were made. In this diagram the symbols have the following meaning. As in § V, a and b are positions on the surface of the plant organ with a near the meristematic region and b some 5–10 mm. away from it. a' and b' are positions near the axis of the organ beneath a and b respectively.

An examination of Fig. 4A shows that the *average* state of affairs in maize roots and turnip hypocotyls is as follows:

(i) The inside of the organ is negative with respect to the outside at any plane between a and b.

(ii) The magnitude of $V_{aa'}$ is greater than that of $V_{bb'}$. That is, the section of the hypocotyl or root at aa' (the plane of the apical meristem) has the larger p.d. between the surface and axis.

(iii) $V_{a'b'}$ is small; b' is usually slightly positive with respect to a'.

Thus, the distribution of potential is consistent with a conducting path following the course of the central tissue of the plant. It is inferred that there is a current flowing inside the plant organs around the circuit $abb'a'a$ due to the larger e.m.f. generated between a' and a, against the smaller e.m.f. between b' and b. Resistance measurements have substantiated this and indicate that this current is probably largely dependent on the moisture condition of the outside of the plant.

* Throughout this paper the sign of the recorded potential gives the polarity of the second suffix symbol with respect to the first, i.e. '$V_{ab}/l_{ab} = -7{\cdot}1 \pm 3{\cdot}8$' means that b is negative to a. The standard deviation given refers throughout to the variation amongst samples and not to experimental error.

(2) *Method of experiment*

Maize roots, Experiment i

Two contact tips were employed, these being placed in agar blobs on the surface of the root at *a* and *b*. The root was immersed in paraffin oil to conserve constant external conditions during measurement. A reading with the tips in this condition gave V_{ab}. The tip at *a* was then pushed into *a'* and time allowed to elapse until the voltage between the tips had assumed a relatively stable value. In certain cases this

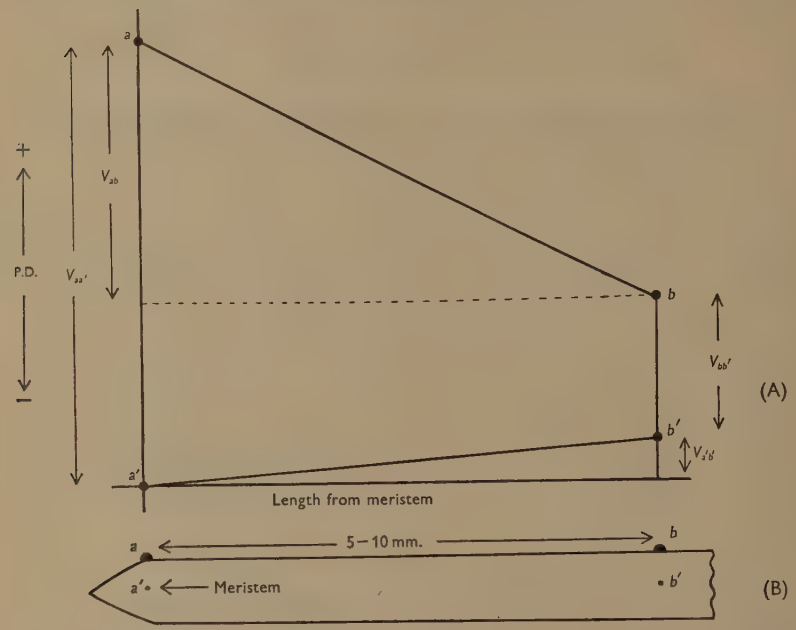


Fig. 4. A. Diagram showing longitudinal and transverse p.d.'s in the neighbourhood of apical meristems. Potential is plotted against length from meristem with *a'*, the interior of the meristem, as origin. *a* represents a point on the surface of the plant at meristematic region, *b* is on the surface 5–10 mm. from *a*, and *b'* is a point on the axis of the plant below *b*. B. Diagram showing the relation of the positions *a*, *b*, *a'*, *b'* to the geometry of the organ (a root is shown).

value was close to the initial reading when the tip was first pushed in. It is regarded as likely in these cases in which the initial reading was the stable one that damage and subsequent injury current flow was at a minimum. Also, in these cases only was the surface p.d. V_{ab} very nearly the same after the experiment as before. In other cases it took a considerably longer time to obtain a stable reading, and this was very different from the initial reading. A large difference in V_{ab} before and after the experiment correlated with this; when such conditions were encountered the experiment was discarded.

The difference between V_{ab} and the stable value of $V_{a'b}$ gave $V_{aa'}$; similarly, $V_{bb'}$ was obtained as the difference between $V_{a'b}$ and $V_{a'b'}$, the latter being the stable reading when the tip at b was pushed to b'.

The experiments in which V_{ab} (after) was within ± 4 mV. of V_{ab} (before) are recorded in Table 1 and are seen to form a consistent pattern, i.e. that of Fig. 4A.

Table 1. *Longitudinal and transverse p.d.'s in 3–5-day maize roots under paraffin oil*

Readings in mV. Sign gives polarity of second suffix symbol with respect to first. *aa'* is in the plane of the meristematic region. See maize roots, method of Exp. i, § VI.

Plant no.	V_{ab}	$V_{aa'}$	$V_{bb'}$	$V_{a'b'}$
1	−22·0	−37·0	−13·0	+2·0
2	−22·0	−35·0	−11·0	+2·0
3	−18·0	−36·0	−14·0	+4·0
4	−17·0	−29·0	−10·0	+2·0
5	−17·0	−29·0	−11·0	+1·0
6	−15·0	−39·5	−19·0	+4·5
7	−12·0	−34·0	−20·0	+2·0
8	−7·0	−17·0	−10·0	0
9	−4·0	−33·0	−33·0	−4·0
10	−3·0	−31·0	−29·0	−1·0
Mean	−13·7	−32·1	−17·0	+1·3
Standard deviation (S.D.) (see footnote to § V)	±6·6	±6·0	±7·8	±2·3

Maize roots, Experiment ii

In Exp. ii uncertainties due to the time rate of change of the potential between the tips when one or both were inside the root were partly eliminated by recording $V_{aa'}$ and $V_{bb'}$ as the *initial* difference between the voltages on the surface and inside the root.

Contact tips were placed initially at points *a*, *b* and *c* on the surface of the plant, that at *c* being a fixed reference tip and not penetrating the plant, while the exploring tips at *a* and *b* were subsequently pushed in to the centre one after the other. The positions *a*, *b* and *c* were in order proceeding from *a* at the surface of the meristematic region towards the base of the root. The lengths between *a* and *b*, and *b* and *c* were approximately equal. Small agar blobs were placed at these spots.

Referring to Fig. 4A, V_{ab}, the surface p.d. was obtained as the difference between V_{cb} and V_{ca}. Next, $V_{ca'}$ was obtained by pushing the exploring tip at *a* rapidly into the centre of the root to *a'*. The *initial* difference between $V_{ca'}$ and V_{ca} was taken to be the p.d. which existed between the surface of the root and the axis ($V_{aa'}$) prior to disturbing it, that is, it is assumed that the potential inside the plant is not abruptly changed when the latter is entered. If time is allowed to elapse between entering the plant and reading the p.d., in general the result will be different due to diffusion of the contents of damaged cells. This method of experimenting is justified by the consistent nature of the results, both internally and compared with those of Exp. i. Similarly $V_{bb'}$ was obtained by subsequently pushing the tip at *b* to *b'* on the axis of the root below *b*. The readings of $V_{a'b'}$, the potential drop along the axis of the root,

were the differences between the readings $V_{cb'}$ and $V_{ca'}$. Table 2 gives the results of experiments such as these, and they are summarized in Fig. 4A.

Table 2. *Longitudinal and transverse p.d.'s in 3–5-day-old maize roots*

Readings in mV. Sign gives polarity of second point with respect to first. See maize roots, method of Exp. ii, § VI.

Plant no.	V_{ab}	$V_{aa'}$	$V_{bb'}$	$V_{a'b'}$
1	−49·5	−72·5	−13·5	+9·5
2	−44·0	−72·0	−23·5	+4·5
3	−42·5	−77·5	−21·0	+14·0
4	−38·5	−45·5	−5·5	+1·5
5	−30·0	−54·5	−23·0	+1·5
6	−30·0	−48·0	−18·0	0
7	−17·0	−34·0	−8·0	+9·0
8	−15·5	−32·0	−6·5	+10·0
9	−15·5	−31·5	−12·5	+3·5
10	−14·5	−32·5	−11·5	+6·5
Mean	−29·7	−50·0	−14·3	+6·0
S.D.	±12·8	±17·4	±6·4	±4·3

When the tip at *b* penetrated the plant, the point *a* in most cases was observed to become more positive with respect to *c*. The experiments of Table 2 are those in which the change was less than 2 mV. It is, of course, possible that the process, whatever it is, may have affected both *a* and *c* nearly equally. For this reason the value of $V_{a'b'}$ cannot be regarded as very significant.

Turnip hypocotyls

In this case a much more fragile organ was used, the diameter being about one-third that of the maize roots.

The experiment was similar to Exp. ii described above. Two exploring tips were used, one after the other being pushed rapidly into the centre of the hypocotyl and the initial changes in potential being read. The 'settled down' voltages did not form a consistent structure, due, as pointed out earlier, to injury effects.

The justification for choosing one set of results rather than the other to represent the static potential distribution inside turnip hypocotyls is found (i) in the consistency of the results (Tables 3A and B), (ii) in the fact that these results suggest a pattern similar to that given by maize *with both types of experiments*, and (iii) the likelihood of damage to such fragile material.

It was realized that when one tip is pushed into *b'*, for example, the potential of *a'* relative to *b'* may be changed by a transient impulse. In addition, the tip now at *b'* does not serve as a fixed reference point for the other tip when it is pushed into *a'* (compare Exp. ii with maize) because of the injury currents flowing near the point *b'*. The two sets of experiments recorded in Tables 3A and B were designed to eliminate these effects as far as possible by altering the order in which the tips are pushed into the interior of the plant. The significant differences between the two sets of figures are explicable by these causes, but it will be noted that the principal features of the observations are the same in each case.

In one set of twenty experiments on the hypocotyls of 3-day-old turnip seedlings, of which ten are given here, the tip at *a* entered before that at *b* and in the other, vice versa. The averages are different, and in particular the value of the p.d. $V_{a'b'}$ can only be an indication of the state of affairs in the unstimulated plant.

Table 3. *Longitudinal and transverse p.d.'s in 3-day-old turnip hypocotyls*

Readings in mV. Sign of voltage gives polarity of second suffix symbol with respect to first. *aa'* is in the plane of the meristematic region.

(A) *Tip at a entered first*

Plant no.	V_{ab}	$V_{aa'}$	$V_{bb'}$	$V_{a'b'}$
1	−47·5	−85·0	−36·5	+1·0
2	−40·0	−87·5	−41·5	+6·0
3	−36·0	−78·5	−38·5	+4·0
4	−34·0	−60·0	−17·0	+9·0
5	−32·0	−72·5	−47·5	−7·0
6	−29·0	−66·5	−31·5	+6·0
7	−27·5	−84·5	−55·0	+2·0
8	−22·5	−65·5	−46·0	−3·0
9	−17·5	−65·0	−37·5	+10·0
10	−7·0	−50·0	−42·0	+1·0
Mean	−29·3	−71·5	−39·3	+2·9
S.D.	±10·9	±11·7	±9·7	±4·7

(B) *Tip at b entered first*

Plant no.	V_{ab}	$V_{aa'}$	$V_{bb'}$	$V_{a'b'}$
1	−49·0	−98·5	−43·5	+6·0
2	−41·0	−102·5	−50·5	+11·0
3	−37·0	−85·0	−55·0	−7·0
4	−37·0	−100·0	−60·0	+3·0
5	−33·0	−82·5	−36·5	+13·0
6	−32·0	−97·5	−55·0	+10·5
7	−25·0	−90·0	−57·5	+7·5
8	−22·5	−68·5	−40·0	+6·0
9	−15·0	−43·5	−17·5	+11·0
10	−12·0	−60·0	−42·5	+5·5
Mean	−30·4	−82·8	−45·8	+6·7
S.D.	±11·0	±18·6	±12·6	±5·4

VII. DISCUSSION

The above work was planned as a step towards the understanding of the role of electric fields in living matter during its growth and organization. An examination of literature and early work in the laboratory showed that research was necessary into the distribution of e.m.f.'s in the region of apical meristems. Even in this simple case the results published and observed were surprisingly erratic. Several slightly different types of experiment are described, designed to allow for or eliminate the many uncertainties encountered in attempting to measure the potential of inside regions of plants relative to their outsides.

The absolute values of the radial p.d.'s measured as described are dependent on the concentration and nature of ions in the contacting fluid and in the contact tips,

on the extent of short-circuiting between outside and inside and on the difference in diffusion potential between contact tip and fluid when outside and between tip and cell sap inside the plants. However, there is enough consistency in the results which show a larger p.d. at the meristematic region than elsewhere to give a basis for the theory, mentioned in the introduction, that meristematic tissue is electrically negative to differentiated tissue surrounding it, in a simple apex.

Details of the distribution of potential between the surface of the organs and their axes has still to be investigated, a large part of the radial e.m.f.'s being thought to be at the interface between exodermis (or epidermis) and contact fluid.

VIII. SUMMARY

1. A research is described using different methods of experiment on two contrasted types of apical meristems with the object of measuring the permanent electrical field inside and outside the plant in the neighbourhood of the apical meristem under conditions of specified control.

2. Most of the experiments were performed on 3–5-day-old maize seedling roots and turnip seedling hypocotyls. The conditions of measurement were such as to produce minimum stimulation of the plant. The general results were checked by experiments with other plants.

3. The measured p.d. is a result of e.m.f.'s which are unchanged when supplying current of the order of 10^{-8} amp. through external conductors. Both turnip hypocotyls and sections of couch grass stems generate power of the order of 10^{-9} W. without polarizing (see text). In these experiments the equivalent resistance of the plant materials obeyed Ohm's law.

4. The average potential gradient along the surface of thirty-four turnip hypocotyls in the neighbourhood of the apical meristems was found to be $7{\cdot}1 \pm 3{\cdot}8$ mV./mm. (over 6–8 mm.). The surface of the meristematic region is positive to that of older tissue. The corresponding average for twenty-five maize roots was $5{\cdot}7 \pm 2{\cdot}4$ mV./mm. (over 6–8 mm.).

5. There are transverse e.m.f.'s between the outside and inside of the plants distributed radially from the stele to the root exodermis and hypocotyl epidermis. The inside is always negative with respect to the outside. The radial e.m.f. is larger in the meristematic region than elsewhere:

In turnip hypocotyls, the radial e.m.f. at the meristematic region varies between 44 and 103 mV.; 5–10 mm. from the apex it is 17–60 mV. In maize roots, the corresponding figures are 17–77 and 6–33 mV.

There is a small but possibly not significant potential change along the axis in both the organs considered, possibly 1 mV./mm. Under the conditions of the experiment, the interior of the meristem was found to be negative with respect to the rest of the axis. Points 4 and 5 are summarized in Fig. 4A.

6. The theory that the interior of a mass of meristematic tissue is electrically negative to older tissue is discussed in the light of the experimental evidence and with regard to the limitations of the technique used.

This work has been done at the Biophysics Laboratory of the University by members of the research staff and of the staff of the Commonwealth Scientific and Industrial Research Organization. We wish to thank C.S.I.R.O. for its support of this work.

REFERENCES

Clark, W. G. (1937). Polar transport of auxin and electrical polarity in coleoptile of *Avena*. *Plant Physiol.* **12**, 737–54.

Lund, E. J. & collaborators (1947). *Bioelectric Fields and Growth*. Austin, Univ. Texas Press. 391 pp.

Lundegårdh, H. (1940). Investigations as to the absorption and accumulation of inorganic ions. *Ann. Agric. Coll. Sweden*, **8**, 233–404.

Nielsen, C. E. (1947). Measurements of small currents: Characteristics of Types 38, 954, and 959 as reduced grid current tubes. *Rev. Sci. Instrum.* **18**, 18–31.

Ramshorn, K. (1934). Experimentelle Beiträge zur electro-physiologischen Wachstumstheorie. *Planta*, **22**, 737–66.

Thomas, J. B. (1939). Electrical control of polarity in plants. *Rec. Trav. bot. néerl.* **36**, 373–437.

CARBONIC ANHYDRASE ACTIVITY DURING EMBRYONIC DEVELOPMENT

By A. M. CLARK

From the Molteno Institute, University of Cambridge, and the Department of Zoology, University of Melbourne

(*Received* 4 *January* 1951)

(With Five Text-figures)

INTRODUCTION

The enzyme carbonic anhydrase, which catalyses the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$, is known to play an important part in the transport of carbon dioxide in the blood of higher vertebrates (Meldrum & Roughton, 1933; Roughton, 1935, 1943; Keilin & Mann, 1941). It is also concerned in the regulation of disturbances in acid-base equilibrium associated with the production of large quantities of hydrochloric acid by the gastric mucosa (Davies, 1948). As it is becoming increasingly evident that this widely distributed enzyme is one of the most important single factors enabling rapid adjustments to be made in acid-base regulation, it is desirable that we should have some knowledge of its formation and physiological importance during embryonic development.

Meldrum & Roughton (1933) observed that carbonic anhydrase does not appear in the circulating blood of the goat foetus until just prior to birth. In human blood taken from the umbilical cord immediately after birth, the enzyme activity is only about one-half that of adult blood (Van Goor, 1934), while still lower values may be observed for the bloods of prematurely born infants (Stevenson, 1943). Van Goor (1940) has reported that there is no carbonic anhydrase in the blood of the developing chick until the twelfth day of incubation, although the enzyme is present in the optic vesicles as early as the third day. Ashby (1943) found very low activity in the blood of the foetal rat, but in a more recent paper (Ashby & Butler, 1948) it is stated that foetal cattle appear to be exceptional in that the blood contains a relatively high concentration of the enzyme.

Van Goor's observation that the enzyme is present at such an early stage in the formation of the eye indicates that carbonic anhydrase is probably synthesized more or less independently in each of the tissues in which it occurs in the adult. Although some observations were made on the carbonic anhydrase activity of several embryonic organs, the chief aim of the present investigation was to ascertain whether the changes in the site of formation of the blood cells bring about any corresponding variations in the amount of enzyme contained within the erythrocytes. The specific points considered, therefore, are the following. At what stage in development is carbonic anhydrase formed in the blood? Is it present in both primitive and

definitive series of red cells? In which haematopoietic tissues is it formed and what changes in activity take place in relation to increase in body size and in the haemoglobin content of the blood? The observations described below enable answers to be given to these questions.

METHODS AND MATERIALS

Carbonic anhydrase activity was determined manometrically at 0° C. by the boat method of Meldrum & Roughton. One side of the boat contained 2·0 ml. of 0·186M-sodium bicarbonate dissolved in 0·038M-sodium hydroxide (Hodgson, 1936), while the other contained 1·0 ml. of 0·4M-phosphate buffer of pH 6·8 and distilled water or enzyme solution to make up a final fluid volume of 4·0 ml. The enzyme unit adopted was that amount of carbonic anhydrase which doubled the initial rate of evolution of carbon dioxide, in μl. per sec., at 0° C., pH 6·8 and a shaking rate of 350 cyc./sec. Precautions were taken to ensure that diffusion did not constitute a limiting factor (Clark, 1949).

Haemoglobin was estimated by Szigeti's method (1940), using the Hilger-Spekker absorptiometer. When the concentration of the pigment was very low, as for example in the homogenates, the more sensitive pyridine haemochromogen method was used in conjunction with a micro-spectroscope and double-wedge trough (Elliott & Keilin, 1933). Blood from chick embryos and foetal mice was taken from the heart or umbilical vessels by means of a capillary pipette. All homogenates were prepared in distilled water.

To enable comparisons to be made between the activities of blood samples containing different numbers of corpuscles, two ratios were calculated. The first, A/Hb, is the number of enzyme units per μl. of whole blood divided by the haemoglobin concentration of the blood in grams per cent. Any change in either the amount of carbonic anhydrase in the red cell or in the corpuscular haemoglobin may affect the value of A/Hb. The second ratio, CA, is the number of enzyme units per million red cells and can be used to distinguish between variations which are actually due to changes in the amount of enzyme contained within the red cells and those merely due to differences in the red cell count.

THE ACTIVITY OF THE BLOOD

Confirming Van Goor's observation, the blood of the developing chick embryo was found to contain no carbonic anhydrase until about the twelfth day of incubation. On the fourteenth day the enzyme is present in appreciable amounts and increases rapidly until the adult level is reached by the eighteenth day. The changes in the haemoglobin content of the blood and in the red cell count which occur during this period are shown in Figure 1, each point representing the mean of three observations. The cell count increases enormously between the fifteenth and nineteenth days but there is a marked fall just before hatching. This increase is reflected by a similar increase in the carbonic anhydrase activity. If, however, we consider not the apparent enzyme activity of the blood but the ratio A/Hb, then it is evident

(Fig. 2) that the formation of carbonic anhydrase in relation to haemoglobin follows a smooth sigmoid curve. The ratio between the two proteins which is characteristic of the blood of the adult is not reached until after hatching.

The physiological significance of the increase in carbonic anhydrase activity during the last period of development has been pointed out by Van Goor and by

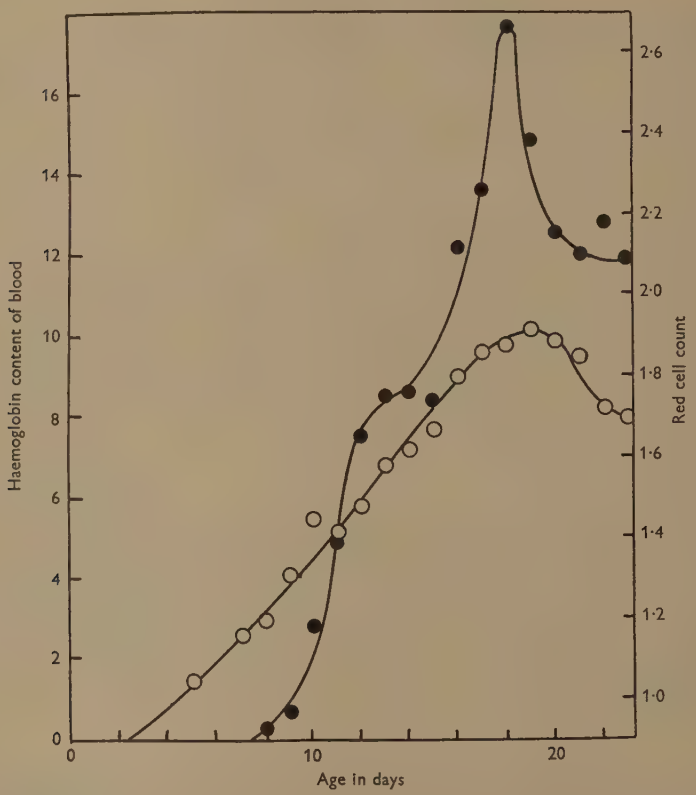


Fig. 1. Haemoglobin content and red cell count in the blood of the developing chick embryo. Open circles = haemoglobin in grams per cent. Closed circles = red cells in millions per cu.mm.

Needham (1942). It is of interest to note that during this period there is a sharp rise in the carbon dioxide output curve for the whole egg (Noyons & De Hasselle, 1939).

Having established the time at which the enzyme appears in the blood, the next point to consider is whether it is confined to any particular series of erythrocytes or whether it is present in all of them. Fig. 3 shows the changes which take place in the value of CA as development proceeds. It is clear that the enzyme must be

entirely absent from the primitive line of red corpuscles, for there is little or no production of these cells after the fifth day, although according to Dawson (1936) a few may persist in the circulation until 2 weeks after hatching. They represent only a very small fraction of the total red cells at the time when carbonic anhydrase activity is first observed in the blood. The conclusion must be, therefore, that the blood islands, blastoderm and endothelial lining of the vascular system are not regions where carbonic anhydrase is formed.

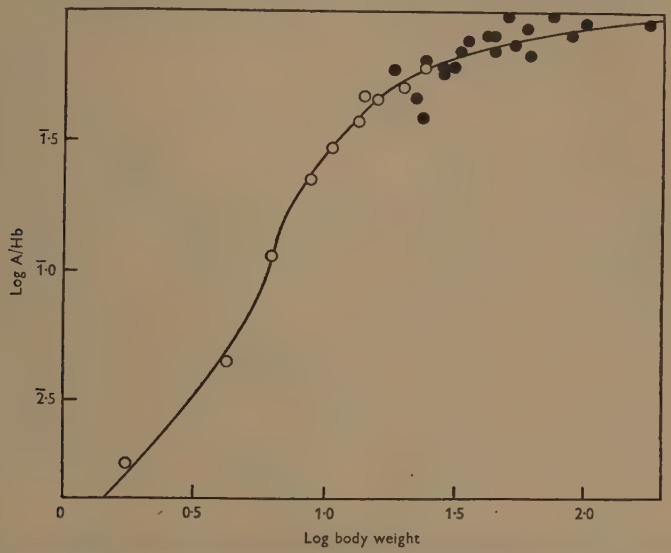


Fig. 2. The ratio between carbonic anhydrase activity, A, and haemoglobin content, Hb, as a function of body weight in the developing chick. A double logarithmic scale has been used to enable a wider range of numerical values to be covered. Open circles = prehatching stages. Closed circles = newly hatched chicks.

The first of the definitive cells likewise appear to lack the enzyme, for they already make up the greater portion of the red cell population by the sixth day. From this time onwards, there is a steadily increasing degree of haemopoietic activity, first of all in the spleen, then in the walls of the yolk sac and finally in the bone marrow. The curve in Fig. 3 shows that the onset of blood formation in the marrow coincides with the increase in carbonic anhydrase content of the corpuscles, although the participation of the other organs is not entirely excluded for there is considerable overlapping. The yolk sac in particular is quite active until about the fourteenth day. However, the fact that the blood-forming activity of the bone marrow increases at the same time as the enzyme activity of the blood, while during the same period the other organs show a gradual decline in their haemopoietic functions, does seem to favour the interpretation that the relatively sudden appearance of carbonic anhydrase in the blood is due to the onset of haemopoiesis in the bone

marrow. It is therefore of some interest to consider whether bone marrow itself shows any enzyme activity.

The femurs of advanced embryos (15–21 days) were removed and split longitudinally. The marrow from one bone was homogenized and used for the estimations of carbonic anhydrase and haemoglobin. The corresponding femur from the opposite side was placed in a small tube containing a known volume of diluting fluid and the marrow scraped out as completely as possible. The bone was transferred to a second

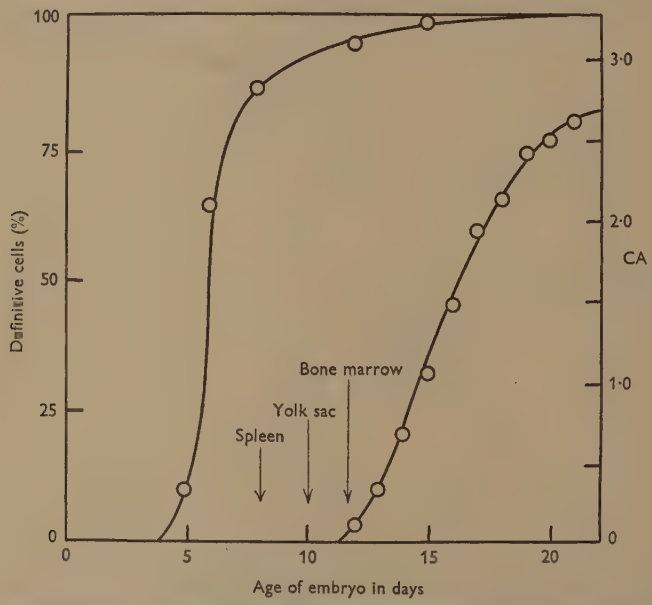


Fig. 3. Carbonic anhydrase activity of chick red corpuscles during development. Left-hand curve and ordinate = the percentage of definitive red cells in the circulating blood. Right-hand curve and ordinate = the number of enzyme units per million red cells. Along the abcissa, the approximate times at which haemopoiesis commences in three organs are indicated by arrows.

tube of fluid and washed for several minutes, the two portions of fluid then being combined quantitatively. This was readily accomplished by the use of wax-coated glassware. The suspension was mixed thoroughly and the number of cells present counted with a haemocytometer. Smears were also made and the proportions of mature and immature red cells determined.

Some typical results are shown in Table 1. There is an enormous increase in the haemopoietic activity of the marrow just before and immediately after hatching. On the other hand, there is a definite fall in the red cell count of the circulating blood during this period. As it is not very probable that the total blood volume rises very much between the nineteenth and twenty-first days, this can only mean that there is an active replacement of erythrocytes at the time of hatching.

Table 1. *Carbonic anhydrase and haemoglobin in the blood and bone marrow of the developing chick*

(A/Hb is the enzyme activity in units per cu.mm. divided by the haemoglobin concentration in grams per cent. Each set of figures the mean of three observations.)

Age of embryo in days	Bone marrow			Blood	
	Red cell count $\times 10^6$	Percent. of mature cells	A/Hb	Red cell count $\times 10^6$	A/Hb
15	14	13	5·2	1·84	0·27
17	35	8	1·7	2·25	0·48
19	36	40	17	2·36	0·59
Hatching					
2	96	12	10	2·12	0·62
4	170	28	32	2·51	0·67
8	780	31	45	2·39	0·71

It may also be noted from Table 1 that the values of A/Hb for bone marrow are always much higher than the corresponding values for the circulating blood. This finds its explanation in the fact that very high values of A/Hb are characteristic of immature erythrocytes, a point that may be demonstrated by increasing the proportion of immature cells in the circulation through severe haemorrhage (Van Goor, 1943; Clark, unpublished). It is not yet clear whether the enzyme is synthesized within the corpuscle before haemoglobin, or whether it passes through a maximum as the cell matures. Either possibility could account for a high value of A/Hb. If, on the other hand, the haemoglobin is being formed in the absence of carbonic anhydrase, then A/Hb will tend to be very low. Comparison of the ratios for blood, bone marrow and yolk sac thus suggests that the latter produces red corpuscles which are devoid of the enzyme (Table 2). It may be argued that the comparison

Table 2. *The ratio of carbonic anhydrase activity, A, to haemoglobin content, Hb, in the blood, bone marrow and yolk sac of the 15-day-old chick embryo*

(The mean of three observations.)

Body weight (g.)	A/Hb, blood	A/Hb, bone marrow	A/Hb, yolk sac
11·2	0·27	5·2	0·08

of values of A/Hb for blood with those for homogenates is not valid, in so far as the first case concerns a population of cells more or less identical, whereas the tissues contain not only erythrocytes but granular leucocytes in various stages of differentiation, as well as the endothelial cells lining the blood sinusoids. This objection is not as serious as might at first appear, for not only do the erythroblasts and erythrocytes greatly outnumber the other cell types present, but they are also the only ones known to possess very high carbonic anhydrase activity. Leucocytes do not contain the enzyme, even though they appear to have a relatively high zinc content (Vallee & Gibson, 1948). It is reasonable to conclude, therefore, that the carbonic anhydrase activity of bone marrow is due almost entirely to the red cells present.

Owing to the greater difficulty in obtaining sufficient blood, the observations on developing mice are not as complete as those on chicks. Nevertheless, a fairly clear picture can be presented of the main changes occurring during development. No carbonic anhydrase appears in the foetal blood until about the fifteenth day (Table 3).

Table 3. *The formation of carbonic anhydrase and haemoglobin in the blood of foetal mice*

(A = enzyme units per cu.mm. of whole blood, Hb = haemoglobin in grams per cent; RBC = the red cell count in millions per cu.mm.; CA = enzyme units per million red cells; CHb = corpuscular haemoglobin in mμg.; V = mean corpuscular volume in cu.μ. All figures are the means of from five to seven observations.)

Age (days)	Hb	A	A/Hb	RBC	CHb	CA	V
13	2·4	—	—	0·37	65	—	220
15	4·8	0·2	0·04	0·92	52	0·22	185
17	6·9	0·7	0·10	1·76	40	0·41	150
21	10·9	1·9	0·17	3·78	30	0·51	117

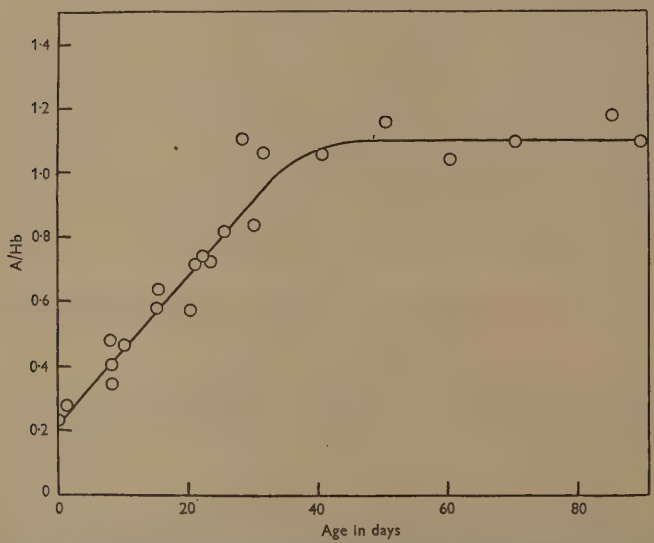


Fig. 4. The ratio between carbonic anhydrase activity, A, and haemoglobin content, Hb, of blood in young mice as a function of age.

The actual value of A/Hb or of CA at any given stage is subject to considerable variation, even within litter-mates, but this variability tends to be reduced in older foetuses. It may be due in part to small individual differences in the times at which the various haemopoietic tissues assume functional importance.

Contrary to the situation in the chick, the carbonic anhydrase activity of mouse blood does not reach maximum levels until several weeks after birth (Fig. 4). This

is presumably a reflexion of the comparatively short gestation period. The relative slowness with which the various haematological characteristics approach steady values (Table 4) suggests that the replacement of red corpuscles, which is believed to take place at birth in the mammal (Smith, 1932; Wintrobe & Shumacher, 1937), proceeds rather slowly in this species.

Table 4. *Carbonic anhydrase and haemoglobin in the blood of young mice*

(Symbols as in Table 3. Each horizontal set of figures was obtained from a single individual)

Age (days)	A	Hb	A/Hb	RBC	CA	CHb	V	CA/V	CHb/V
Newborn	2·5	11·6	0·22	3·61	0·71	32	92	0·008	0·348
2	3·8	12·5	0·30	4·02	0·94	31	88	0·016	0·352
7	5·3	11·7	0·52	5·01	1·04	23	75	0·014	0·306
14	7·5	12·7	0·60	5·76	1·32	22	69	0·019	0·318
15	9·2	12·4	0·74	6·28	1·46	20	67	0·027	0·299
18	6·0	10·7	0·56	5·67	1·06	19	62	0·018	0·306
20	7·1	9·7	0·73	5·80	1·22	17	55	0·022	0·309
22	7·6	10·1	0·75	5·78	1·32	17	52	0·025	0·327
25	10·9	12·4	0·87	7·04	1·55	18	60	0·026	0·300
26	12·4	10·6	1·17	6·74	1·84	16	58	0·032	0·276
28	11·0	10·2	1·10	6·75	1·63	15	59	0·028	0·259
50	18·2	15·3	1·20	10·2	1·78	15	52	0·034	0·288
60	16·5	15·5	1·06	9·56	1·73	16	54	0·032	0·296
90	15·5	15·0	1·03	9·41	1·64	16	53	0·031	0·302

CARBONIC ANHYDRASE IN EMBRYONIC TISSUES

The only published observations on the carbonic anhydrase activity of embryonic tissues are those of Van Goor (1940) and of Ashby & Butler (1948), both of which deal more particularly with parts of the nervous system. It has already been mentioned that Van Goor found the enzyme to be present in the optic cup of the 72 hr. chick. Ashby & Butler, working on foetal cattle and prematurely born human infants, find that in the central nervous system the adult pattern of distribution of the enzyme is reached by the beginning of the last quarter of gestation. The cerebrum appears to be exceptional in that no carbonic anhydrase activity is apparent until just before birth, in the case of cattle, and not until after birth in the case of humans. In view of its relatively late appearance in the developing central nervous system, carbonic anhydrase differs from other enzymes that have been investigated. Succinic dehydrogenase, cytochrome oxidase and choline esterase, for example, all appear at an early stage, the rise in their activities being closely associated with increasing morphological complexity (Nachmansohn, 1940; Youngstrom, 1941; Flexner, Flexner & Straus, 1941, 1946). These enzymes are probably of far greater importance for the metabolism and functional activity of nervous tissues. It may be noted that Davenport (1946) has shown that carbonic anhydrase plays no essential role in the activity of nerve fibres.

In the present investigation, the organs selected for study were the lens, retina, stomach, kidney and brain. Corrections for the enzyme activity of traces of blood present in the homogenates were made by measuring the concentration of haemo-

globin, using the pyridine haemochromogen method, and calculating the value of A/Hb for both tissue and blood.

Carbonic anhydrase develops more or less independently in the different organs and at quite different rates (Fig. 5). Only in the stomach does the increasing activity occur at about the same time as that observed in the blood. However, the most striking feature is the way in which the enzyme may be formed in the organs long before the onset of functional activity. As Barcroft (1934) remarked in another connexion, 'the stage is set before the play commences'. The retina is the extreme

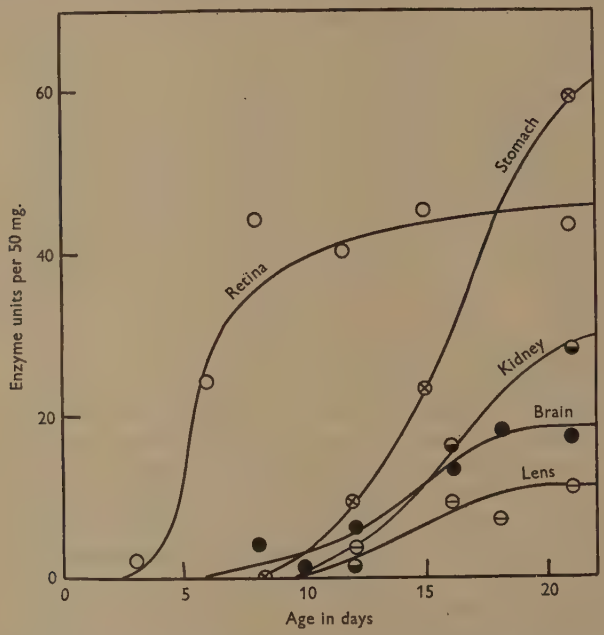


Fig. 5. The carbonic anhydrase activity of various organs at different stages in the development of the chick embryo. Ordinate = enzyme units per 50 mg. of wet tissue.

case of this. Bakker (1939) and Leiner (1940) have suggested that the high rate of glycolysis of the vertebrate retina necessitates the presence of a large amount of carbonic anhydrase to facilitate the removal of carbon dioxide, but there would appear to be no real evidence to support this, since it has yet to be shown that the non-enzymically catalysed rate for the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$ at 38° C. is inadequate for physiological requirements. In any case, it is perhaps open to doubt whether the presence of the enzyme in large amount would in fact facilitate the removal of carbon dioxide, for it would convert rapidly diffusing gas molecules into the more slowly diffusing bicarbonate ions.

It is rather difficult to assess the amount of functional activity which takes place

in the other organs before birth. According to Gundobin (1912), as soon as gastric glands become recognizable histologically they can secrete acid, but this has been denied by Schmidt (1914), who claims that no acid is produced until after birth. Sutherland (1921), however, found convincing evidence for the secretion of acid in the foetal guinea-pig stomach, which suggests that gastric carbonic anhydrase is already playing a role in acid-base regulation (Davies, 1948). There is also evidence to show that a considerable amount of renal activity takes place before birth (Needham, 1942; McCance, 1948), in which the enzyme undoubtedly participates, but accurate information for either the chick or the mouse is lacking.

DISCUSSION

The changes which occur in the values of A/Hb and CA during the latter period of development in the chick embryo are of considerable interest in connexion with the work of Hall (1934). He found that from about the tenth day of incubation until 3 weeks after hatching there is a gradual shift in the dissociation curve of chick haemoglobin, indicating a decrease in the affinity of the pigment for oxygen. Hall explains this by supposing that an embryonic type of the pigment is slowly replaced by an adult type. As Dawson (1936) points out, this cannot be due to the replacement of the primitive red cells by those of the definitive line, for the shift in the dissociation curve is most marked between the twelfth and eighteenth days. On the other hand, it is significant that the shift coincides closely with the appearance of carbonic anhydrase activity in the blood and with the onset of haemopoiesis in the bone marrow. Dawson has suggested that the shift in the dissociation curve is due to the replacement of red cells formed in the yolk sac by those formed in the bone marrow. Now the values of A/Hb given in Table 2 indicate that the corpuscles originating in the yolk sac lack carbonic anhydrase. Therefore, if we accept Dawson's suggestion that these cells contain a haemoglobin different to that produced in marrow, it is then possible to explain the fact that the rapid shift in the dissociation curve, the increase in carbonic anhydrase activity and the growing importance of bone marrow as a haemopoietic tissue all occur at the same time. The replacement of corpuscles which is assumed to take place (Table 1) would fit in perfectly with this picture. It may be recalled that Smith (1932) found evidence indicating that there is a complete change in the blood cell population of the rat at birth. The results of the present observations suggest that what actually occurs is the replacement of a large foetal type of corpuscle, containing the embryonic type of haemoglobin but no carbonic anhydrase, by a smaller type of cell in which the enzyme is present together with the adult haemoglobin. Recently, Jonxis (1948) has also suggested that foetal and adult haemoglobins do not occur together in the same corpuscle. He claims that in erythroblastis foetalis the corpuscles containing foetal haemoglobin are broken down preferentially, while those containing the adult type are more or less unaffected.

Using the method of alkali denaturation, Brinkman & Jonxis (1936) have shown that the relative proportions of foetal and adult haemoglobins present in blood can be ascertained. If it could be demonstrated that the replacement of the foetal type

of haemoglobin commences at the same time that carbonic anhydrase activity appears, it would be strong evidence that a new kind of erythrocyte is put into circulation at that stage of development. In any case, it is difficult to see how one can explain the gradual increase in CA and A/Hb over a period when the red cell count may show sudden changes, except in terms of a mixed population of cells in which active replacement is proceeding.

SUMMARY

1. Tissue carbonic anhydrase is usually formed at an early stage in the embryonic development of the chick and mouse. The enzyme does not appear in the blood until a relatively late stage has been reached.

2. In the erythrocytes, it is probable that the enzyme is confined to those cells produced in bone marrow.

3. Evidence is presented to support the theory that towards the end of development, there is a replacement of red cells which contain an embryonic type of haemoglobin but no carbonic anhydrase, by corpuscles in which the enzyme is present together with the adult type of haemoglobin.

This work was carried out during the tenure of an 1851 Exhibition Science Research Scholarship. My thanks are due to Prof. D. Keilin, F.R.S., for his interest in the problem, and to Dr P. Tate for providing some of the material.

REFERENCES

ASHBY, W. (1943). *J. Biol. Chem.* **151**, 521.
ASHBY, W. & BUTLER, E. (1948). *J. Biol. Chem.* **175**, 425.
BAKKER, A. (1939). *V. Graefs Arch. Ophthal.* **140**, 543.
BARCROFT, A. (1934). *Features in the Architecture of Physiological Function.* Cambridge University Press.
BRINKMAN, R. & JONXIS, J. H. P. (1936). *J. Physiol.* **88**, 162.
CLARK, A. M. (1949). *Nature, Lond.*, **163**, 562.
DAVENPORT, H. W. (1939). *J. Physiol.* **97**, 32.
DAVENPORT, H. W. (1946). *J. Neurophysiol.* **9**, 41.
DAVIES, R. E. (1948). *Biochem. J.* **42**, 609.
DAWSON, A. B. (1936). *Z. Zellforsch.* **24**, 256.
ELLIOTT, K. A. C. & KEILIN, D. (1933). *Proc. Roy. Soc.* B, **114**, 210.
FLEXNER, J. B., FLEXNER, L. B. & STRAUS, W. L. (1941). *J. Cell. Comp. Physiol.* **18**, 355.
FLEXNER, L. B. & FLEXNER, J. B. (1946). *J. Cell. Comp. Physiol.* **27**, 35.
GUNDOBIN, B. N. (1912). *Die Besonderheiten des Kindesalters.* Berlin.
HALL, F. G. (1934). *J. Physiol.* **83**, 222.
HODGSON, T. H. (1936). *Brit. J. Exp. Path.* **17**, 75.
JONXIS, J. H. P. (1948). *Nature, Lond.*, **161**, 850.
KEILIN, D. & MANN, T. (1941). *Nature, Lond.*, **148**, 493.
LEINER, M. (1940). *Naturwissenschaften*, **28**, 165.
MCCANCE, R. A. (1948). *Physiol. Rev.* **28**, 331.
MELDRUM, N. U. & ROUGHTON, F. J. W. (1933). *J. Physiol.* **80**, 133.
NACHMANSOHN, D. (1940). *J. Neurophysiol.* **3**, 396.
NEEDHAM, J. (1942). *Biochemistry and Morphogenesis.* Cambridge University Press.
NOYONS, A. K. M. & DE HASSELLE, P. M. H. (1939). *Acta brev. neerl. Physiol.* **9**, 170.
ROUGHTON, F. J. W. (1935). *Physiol. Rev.* **15**, 241.
ROUGHTON, F. J. W. (1943). *Harvey Lect.* **39**, 96.

SCHMIDT, R. (1914). *Biochem. Z.* **63**, 287.
SMITH, C. (1932). *J. Path. Bact.* **35**, 717.
STEVENSON, S. S. (1943). *J. Clin. Invest.* **22**, 403.
SUTHERLAND, G. F. (1921). *Amer. J. Physiol.* **55**, 398.
SZIGETI, B. (1940). *Biochem. J.* **34**, 1460.
VALLEE, B. L. & GIBSON, J. G. (1948). *J. Biol. Chem.* **176**, 445.
VAN GOOR, H. (1934). Bijdragen tot kennis van de koolzuuranhydrase. Dissertation. Groningen.
VAN GOOR, H. (1940). *Acta brev. neerl. Physiol.* **10**, 37.
VAN GOOR, H. (1943). *Acta brev. neerl. Physiol.* **13**, 21.
WINTROBE, M. M. & SCHUMACHER, H. B. (1937). *Amer. J. Anat.* **58**, 313.
YOUNGSTROM, K. A. (1941). *J. Neurophysiol.* **4**, 473.

THE RESPIRATORY EXCHANGE OF THE DESERT LOCUST (*SCHISTOCERCA GREGARIA*) BEFORE, DURING AND AFTER FLIGHT

By the late AUGUST KROGH and TORKEL WEIS-FOGH

From Professor August Krogh's Laboratory, Søbredden 24, Gentofte, Denmark

(With Four Text-figures)

During the last two decades the knowledge of the physiology of heavy muscular work has increased considerably, and the high metabolic rate of flying insects has attracted particular attention. Jongbloed & Wiersma (1935), Chadwick & Gilmour (1940), and Davis & Fraenkel (1940) have reviewed the literature concerning the metabolic rate of flying insects. More recent investigations dealt mainly with the nature of the fuels combusted or with other aspects of insect flight. Most of the experiments referred to below dealt with measurements of the oxygen consumption of insects performing flight movements in a relatively small container. In this respect and because generally the animals were suspended and the natural locomotion was prevented the conditions of flight were abnormal. However, these limitations concerning the conditions of flight are probably of minor importance when we are interested mainly in the order of magnitude of the metabolic rate and in the nature of the fuels combusted, and with these limitations in mind, the present state of knowledge might be summarized:

(1) During flight the oxygen consumption is considerably increased and has been estimated at about 100 l. O_2/kg. body weight/hr. in bees (Jongbloed & Wiersma, 1935), flies (Davis & Fraenkel, 1940), and butterflies (Zeuthen, in Krogh, 1941). This means that the metabolic rate during flight in some insects increases 100 times or even more compared with the metabolism during rest. In an extensive and very interesting series of experiments Chadwick & Gilmour (1940) and Chadwick (1947) have demonstrated that various species of *Drosophila* consumed only 20 l. O_2/kg./hr., a figure which was indirectly verified by Williams, Barness & Sawyer (1943) and Wigglesworth (1949). In the above-mentioned papers by Chadwick, and a paper by Chadwick & Williams (1949), the metabolic rate was correlated with the wing-beat frequency.

(2) The respiratory quotient (R.Q.) during flight was measured in bees (Jongbloed & Wiersma, 1935, in *Apis*) and flies (Chadwick, 1947, in *Drosophila*). In both cases the R.Q. equalled unity, indicating the combustion of carbohydrates, and this interpretation seems to be correct, glucose and glycogen being utilized as fuels in *Apis* (Beutler, 1937) and *Drosophila* (Williams *et al.* 1943; Wigglesworth, 1949) respectively.

(3) After the cessation of flight a small oxygen debt was demonstrated in *Drosophila* (Chadwick & Gilmour, 1940). The debt was abolished in less than 2 min.

August Krogh's unpublished analyses of the R.Q. of flying Lepidoptera gave values about unity, and so it seems reasonable to assume that the higher insects derive the energy for flight movements from the combustion of carbohydrates. Many insects generally considered primitive from a morphological point of view are, nevertheless, excellent flyers in one or more respects. The dragonflies, for example, are second to none as far as the flying speed and the refined regulation of the flight movements are concerned, and locusts are able to endure sustained flight for several hours every day during a considerable period of time. As the migrations of locusts are of great economic importance, it is thus of interest from both practical and theoretical points of view to study the metabolism during flight in these relatively primitive insects. The purpose of the present paper has been to study some quantitative and qualitative aspects of the combustion of flying locusts.

MATERIAL

If not otherwise stated, the animals used for experiments were mature male *Schistocerca gregaria* (Forskål). They resembled the transiens phase in adult biometry and the gregarious phase in hopper coloration. They were bred in crowded cages at the Anti-Locust Research Centre, London, and were sent by plane from London to Copenhagen as young adults, usually less than 7 days after emergence, each individual being placed in a small plastic tube. The animals were undamaged and fresh on arrival. They were kept in cages at 27–30° C. and fed on green cabbage and dried green grass. The mortality was low and both sexes reached sexual maturity in 3–4 weeks after the final moult. A few days later oviposition began, indicating that the food was sufficient. Female locusts, under laboratory conditions, were disinclined to fly when they were loaded with eggs, and so only males were used. The experimental animals were generally taken directly from the cages, but in a few cases they were previously starved up to 72 hr., and in some cases it was tried to select the less active animals in order to facilitate the determination of the metabolic rate during rest.

METHODS

Measurements (to be described in a later paper) of the flying speed and the wing-beat frequency of *Schistocerca* flying in front of a wind tunnel revealed that variations in the temperature of the ambient air between 25 and 35° C. had only a slight effect upon the intensity of flight, in contrast to what Chadwick (1939) found in *Drosophila* in which the wing-beat frequency decreased considerably with decreasing temperature. For this reason we have only endeavoured to keep the temperature of the experimental room within 27–30° C. Some few experiments were performed at a lower (22° C.) or at a higher (35° C.) temperature.

All determinations of the oxygen consumption and the R.Q. were based on gas analyses performed by an analyser of the Haldane type, accurate to about 0·01 %. An air sample of about 20 ml. was drawn from the container and was analysed for CO_2, O_2 and 'N', and the results were calculated according to Krogh (1920). Even when the initial composition of the air in the container might have been taken for

granted it was often, but not always, similarly analysed to correct for systematic variations in the analytical procedure. The double analyses represented in Table 2 (Exps. 1–7) indicate the reliability of the analytical method. The oxygen consumptions have been reduced to N.T.P.

For determinations during rest we adopted the following procedure. In the first series of experiments one or two animals were placed in a relatively large container of 100–200 ml. capacity filled at the desired temperature by a rapid stream of pure but moist atmospheric air. Generally the pressure was raised by 20–40 mm. of mercury above the atmosphere and read at intervals to make sure that the system was airtight. The animal or animals were kept enclosed for a suitable time in order to make the CO_2 content increase approximately to 1%. Control experiments in which the CO_2 percentage was raised artificially to 4–7% failed to show any significant increase of the metabolic rate in spite of the increased rate and depth of the respiratory movements. In other series of experiments on resting animals, we reduced the volume of the container to 20 ml. or less, ventilated by a rapid current of air, started the experiment suddenly by interrupting the ventilation, and connected the container to a gas-sampling vessel filled with mercury. This enabled us to do experiments of 5–15 min. duration which were brought to a stop by sucking practically all the air over into the gas-sampling vessel and replacing it with acidulated water. After such an experiment the animal had to be dried in an air current before it could be used again, but the procedure did not damage the animal in any way. This type of experiment was used especially to study the 'oxygen debt' incurred during flight.

For determination during tethered 'flight', we took advantage of the fact that a suitably suspended locust can be made to maintain flight movements for a long period of time if the aerodynamic sense organ on the upper part of the head is stimulated by a jet of air (Weis-Fogh, 1949, 1950). The locust was suspended from a small metal bar fixed to the prothorax with a sticky wax (10 g. beeswax + 4·5 g. colophony) and placed in the respiration chamber. In order to prevent the tarsi finding any support, the distal parts of the legs were sometimes cut off, and the wounds covered with wax, at least 24 hr. before the experiment. If the tarsi of a locust are able to contact the surrounding walls or even its own wings it stops immediately (cf. Fraenkel, 1932). The respiration chamber consisted of a container in which the air could be circulated by a pump and delivered as a jet directed towards the upper part of the head. The pump (Fig. 1, *A*) consisted of rubber tubing in which the air was driven forward by means of rollers (Krogh, 1904). In the preliminary series of experiments a glass container of 1180 ml. capacity was used, but when it became desirable to do determinations of a few minutes' duration only, we used a box of Plexiglas (Fig. 1) holding at first 517 ml. and later 532 ml., on account of an extension (*B*) partly made of wide rubber tubing distally closed by a rubber plug. This extension made it easier to adjust the air jet. However, CO_2 is slightly soluble in rubber and therefore it became necessary to determine the rate of the disappearance of CO_2 under the experimental conditions. At the CO_2 tensions in question the loss of CO_2 corresponded to 0·005–0·01% per experiment as far as the large and the 517 ml. containers were concerned and so it could be neglected (Table 2, Exps. 1–9),

the accuracy of the gas analysis being 0·02%. But in the 532 ml. container the rubber extension caused a loss corresponding to 0·02–0·05% per experiment, and the values in Table 2, Exps. 10 and 11, have been corrected accordingly.

When successive experiments were performed with the same animal, the pump and the locust continued to work, moist atmospheric air being blown through the container in order to renew the air during the intervals between the experiments (in at *C*, out at *D*). After 2–3 min. of ventilation, a sample of the air in the container was taken for analysis (at *E*) and a new experiment was then started by interrupting the air current. The temperature and the pressure (manometer connected at *F*) were

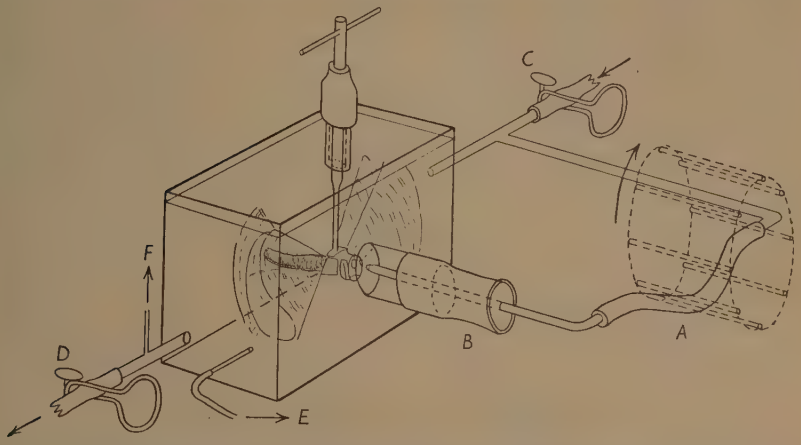


Fig. 1. Respiration chamber with a suspended *Schistocerca* which is stimulated by means of a jet of air to perform flight movements. For further explanation, see text.

controlled at the beginning and at the close of each experiment. During an experiment the percentage of CO_2 was allowed to increase from about 0·05 to about 1%. It was possible to make a locust fly continuously for 1½ hr. when the air jet was properly adjusted.

RESULTS

Before flight

In order to get a rough estimate of the relationship between the metabolic rate in resting locusts and the temperature, a small series of experiments was performed in which mature males were enclosed in a fairly large container for 2–3 hr. at different temperatures. The results are plotted as circles in Fig. 2, in which the metabolic rates are given as litres of oxygen consumed per kg. body weight per hour. It is seen that the metabolic rate at about 30° C. could serve as a reasonable basis of comparison between the exchange during rest and flight since the intensity in resting animals varied roughly linearly with temperatures between 25 and 35° C., i.e. within the range of temperatures (no heat radiation) where sustained flight generally could be

performed in the laboratory. The R.Q. of the insects which were taken directly from the cage averaged 0·82 ± 0·03.

In order to get a representative value for the respiratory exchange in resting animals a series of twenty-three experiments was performed at 27–30° C. Single mature males were taken from the cages and placed in a 20 ml. container for 6–18 min. Table 1 contains the results. As was to be expected, the metabolic rate varied to a considerable degree (Fig. 2, crosses), the average value amounting to 0·63 ± 0·03 l. O_2/kg./hr. (S.D. 0·13) at 28° C. But this accuracy was sufficient for our purpose. The R.Q., on the other hand, was rather constant and averaged 0·82 ± 0·013.

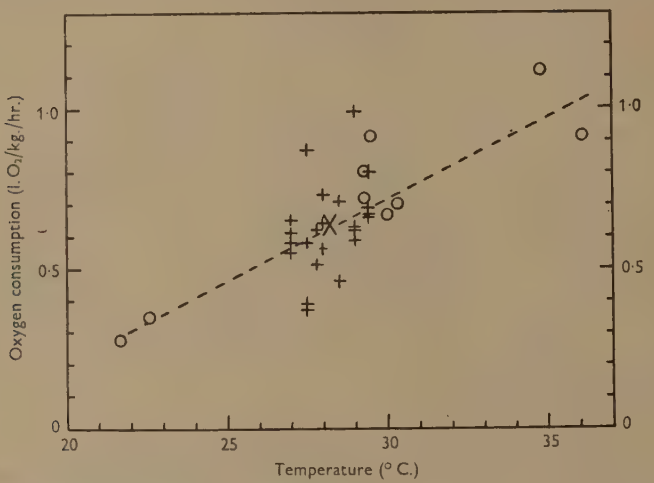


Fig. 2. Oxygen consumption of resting *Schistocerca* males in relation to temperature.

After the experiments the same locusts were made to fly for shorter or longer periods, and after the cessation of flight the respiratory exchange was followed (see later, Table 3).

During flight

Table 2 summarizes eleven experiments (twenty-five measurements) on the respiratory exchange of flying *Schistocerca* males. As in Table 1, the results are given partly as the oxygen uptake in cu.mm. oxygen consumed per animal per minute and partly as l. O_2/kg. body weight/hr. Exps. 1–6 refer to the initial 12–30 min. of flight, whereas Exps. 7–11 show the results obtained during successive intervals of uninterrupted flight, the maximum flying time being 101 min.

Under these artificial conditions one could not expect to get any constant figure for the oxygen uptake during flight, and in fact the oxygen consumption varied between 10 and 30 l. O_2/kg./hr. corresponding to 300–1000 cu.mm. O_2/male/min. The average consumption was 15 l. O_2/kg./hr. This means that the metabolic rate during flight had increased fifteen to fifty times compared with the value during rest.

Table 1. *Respiratory exchange during rest at 27–30° C.*

(Mature *Schistocerca* ♂♂, directly from the cages. Small containers.)

Date (1949)	Animal no.	Weight (g.)	Temperature (° C.)	Duration of experiment (min.)	Respiratory exchange		
					O_2 uptake per animal per min. (cu.mm.)	O_2 uptake (l. O_2/kg./hr.)	R.Q.
15. ii.	III	1·9	27·5	6·42	28	0·87	0·83
15. ii.	VI	1·6	27·5	5·67	10	0·37	0·90
16. ii.	VI	1·6	27·8	11·40	16	0·62	0·84
21. ii.	VI	1·6	28·0	11·55	15	0·56	0·76
23. ii.	VI	1·5	29·4	11·67	17	0·69	0·76
23. ii.	VI	1·5	29·4	17·72	17	0·67	0·74
15. ii.	VII	1·5	27·5	5·25	10	0·39	0·90
16. ii.	VII	1·5	27·8	10·20	12	0·51	0·65
21. ii.	VII	1·5	28·0	10·45	18	0·73	0·84
21. ii.	VII	1·5	28·0	10·40	16	0·64	0·78
23. ii.	VII	1·6	29·4	13·47	21	0·80	0·85
23. ii.	VII	1·6	29·4	14·60	18	0·66	0·84
10. iii.	VII	1·6	27·0	17·43	17	0·65	0·80
10. iii.	VII	1·6	27·0	17·27	15	0·58	0·84
10. iii.	VII	1·6	27·0	17·35	16	0·61	0·79
10. iii.	VII	1·6	27·0	17·32	15	0·55	0·81
25. ii.	X	1·6	29·0	12·22	17	0·62	0·88
25. ii.	X	1·6	29·0	12·78	26	0·99	0·90
15. ii.	XVII	1·4	27·5	6·25	14	0·58	0·90
25. ii.	XVIII	1·5	29·0	12·18	16	0·63	0·83
1. iii.	XVIII	1·5	28·5	14·48	18	0·71	0·79
1. iii.	XVIII	1·5	28·5	15·43	12	0·46	0·74
25. ii.	XXIII	1·8	29·0	13·55	18	0·59	0·88

Oxygen consumption average $0·63 \pm 0·03$ l. O_2/kg./hr. S.D. $= 0·13$.
Respiratory quotient: average $0·82 \pm 0·01$ l. O_2/kg./hr. S.D. $= 0·06$.

Although these figures are of a moderate size compared with the figures for flies and bees, formerly referred to, they are high enough to show that the metabolism was radically altered, and presumably resembled the metabolism during natural flight both as far as rate of combustion and nature of the fuels are concerned. Consequently, a closer analysis of the variations of the R.Q. was of special interest. The experiments, nos. 7–11, revealed that the R.Q. at the end of the period of flight was always less than at the beginning, and if we average the figures obtained during the first 10–40 min. of flight in the experiments in which the animals were taken directly from the cages (twelve measurements) the R.Q. was $0·82 \pm 0·01$, i.e. the R.Q. equalled the value before flight. If, however, we average the values obtained after at least 30 min. of flight we get $0·75 \pm 0·02$. The difference between the two figures, $0·07 \pm 0·02$, is statistically significant, and thus it has been demonstrated that the R.Q. decreased during sustained flight, indicating an increased combustion of fats. No correlation between metabolic rate and R.Q. could, however, be demonstrated.

After flight

It is possible to make a locust fly continuously for many hours under rather natural conditions if it is properly suspended in front of a wind tunnel or if it is attached to a round-about (Krogh & Weis-Fogh, 1951). After sustained flight, the

Table 2. *Respiratory exchange during flight*

(*Schistocerca* ♂♂.)

Exp. no.	Weight (g.)	Temperature (° C.)	Time after the beginning of flight (min.)	Respiratory exchange		R.Q.	Sexual development. Treatment before the experiment
				O_2 uptake per animal per min. (cu.mm.)	O_2 uptake (l. O_2/kg./hr.)		
1	—	34·5	0–11·5	780	—	0·82	Mature. Directly from c
				750	—	0·82	
2	1·9	29·9	0–29·0	600	18·8	0·79	Mature. Directly from c
				620	19·4	0·75	
3	2·0	22·9	0–30·5	490	14·8	0·74	Mature. Has flown for 1 about 3 hr. before experiment
				490	14·9	0·74	
4	2·4	23·0	0–30·0	530	13·4	0·81	Mature. Directly from c
				540	13·8	0·81	
5	2·4	30·2	0–30·0	600	15·1	0·83	Mature. Directly from c
				620	15·6	0·81	
6	2·4	30·2	0–21·5	770	19·2	0·73	Same individual as in Exp after 30 min. of rest
				790	19·9	0·71	
7	2·2	30·0	0–9·5	900	24·8	0·83	Mature. Starved ab
				910	25·1	0·83	20 hr. R.Q. during
		30·2	13–32	1030	28·4	0·77	0·74
				1040	28·6	0·77	
8	1·6	26·5	0–14	390	14·4	0·88	Mature. Directly from c
			19–32	370	13·9	0·85	
			33–49	300	11·2	0·84	
			51–68	280	10·2	0·84	
			71–92	320	11·9	0·82	
9	1·6	27·3	0–18	360	13·5	0·84	Directly from cage. Sa individual as in Exp 4 days later
			21–41	370	13·7	0·77	
			43–61	330	12·5	0·77	
			64–82	340	12·7	0·73	
			84–101	340	12·7	0·78	
10	1·5	28·0	0–19	370	14·7	0·82	Immature. Directly fr cage
			22–43	250	9·9	0·82	
			45–67	310	12·4	0·69	
11	1·6	27·5	0–18	440	16·5	0·79	Immature. Directly fr cage
			20–37	340	12·9	0·84	
			42–64	340	12·9	0·75	
			66–87	380	14·1	0·69	

locusts generally settled quietly where they were placed in the cage, they did not eat unless they were placed directly on the food, and the rate of the respiratory movements was increased. In short, the animals appeared to be tired, although they were able to resume flight when suspended again. In order to study the metabolic rate during this phase of recovery, the same animals as referred to in Table 1 were forced to fly for shorter (10 min.) or longer periods (60–195 min.) by application of either of the methods mentioned above. Immediately after the interruption of flight each animal was placed in a 20 ml. container and the respiratory exchange was followed during successive intervals of time, preferably in the same individual, but in some experiments different animals were used. The results are seen in Table 3. The average values of the oxygen uptake and the R.Q. before flight have been taken from

Table 3. *Respiratory exchange after flight at* 27–30° C.

(Mature *Schistocerca* ♂♂: the same individuals as in Table 1.)

Date (1949)	Animal no.	Respiratory exchange before flight (from Table 1)		Duration of flight (min.)	Time after cessation of flight (min.)	Respiratory exchange after flight	
		O_2 uptake (l. O_2/kg./hr.)	R.Q.			O_2 uptake (l. O_2/kg./hr.)	R.Q.
21. ii.	VI	0·67	0·76	10	0·5–5·5	1·49	0·80
					11–18	0·95	0·82
					25–37	0·70	0·82
					55–66	0·71	0·81
					80–92	0·68	0·74
23. ii.	VII	0·75	0·84	10	1·5–7	1·92	0·72
					27–38	1·15	0·61
					54–66	0·79	0·74
1. iii.	XVIII	0·57	0·77	10	0·5–6	1·34	0·89
					10–19	0·71	0·76
					28–41	0·68	0·66
					52–68	0·49	0·67
15. ii.	VII	0·39	0·90	60	0·5–6	1·27	0·84
	III	0·87	0·83	60	7–12	0·77	0·76
					40–49	0·45	0·73
23. ii.	VI	0·66	0·75	91	1–6	2·10	0·70
					11–18	1·28	0·66
					25–36	1·03	0·69
					48–59	1·00	0·62
					78–90	1·18	0·75
25. ii.	XVIII	0·67	0·85	99	1–6	1·54	0·76
					11–18	0·96	0·83
					23–34	1·02	0·76
					41–54	0·96	0·77
					84–96	0·75	0·71
					128–142	0·74	0·66
21. ii.	VII	0·73	0·81	103	0·5–6	1·93	0·71
					11–19	1·13	0·91
					28–40	0·91	0·73
					46–60	0·81	0·72
					76–90	0·85	0·68
15. ii.	XVII	0·58	0·90	120	1–6	1·14	0·73
	VI	0·37	0·90	120	7–13	1·23	0·73
	XVII	0·58	0·90	120	19–29	0·55	0·85
9. iii.	VII	0·62	0·81	195	0·5–6	1·50	0·73
					12–20	1·23	0·77
					29–45	1·05	0·76
					61–80	0·82	0·70
					101–121	0·77	0·73
					152–169	0·77	0·69
16. ii.	VII	0·51	0·65		1–6	1·11	0·86
	XI				8–15	0·86	0·79
	VI	0·62	0·84		16–24	0·93	0·83
	XXV				25–36	0·70	0·78
16. ii.	X				2–12	1·31	0·79
	XXIII				26–36	0·85	0·70
	III				47–57	0·63	0·71
	XVIII				67–77	0·63	0·71

Table 1, and if we compare these results with the results obtained after even a short period of flight, it is seen that the metabolic rate just after the flight had stopped was increased by two or three times, and furthermore that the recovery lasted at least

Table 4. *The oxygen uptake after flight: the 'oxygen debt'*

Duration of preceding period of flight (min.)	Animal no.	Oxygen uptake in l. O_2/kg./hr. at different times after cessation of flight (min.)					O_2 uptake before flight (l. O_2/kg./hr.)
		3·5	10	20	30	60	
10	VI	1·49	0·94	0·78	0·70	0·70	0·67
10	VII	1·92	1·73	1·46	1·22	0·78	0·75
10	XVIII	1·34	0·92	0·68	0·68	0·48	0·59
Average		1·58	1·20	0·97	0·87	0·65	0·67
91	VI	2·10	1·58	1·18	1·04	1·02	0·66
99	XVIII	1·54	1·07	0·96	1·02	0·91	0·67
103	VII	1·93	1·36	1·04	0·93	0·82	0·73
195	VII	1·50	1·33	1·18	1·09	0·89	0·62
Average		1·77	1·34	1·09	1·00	0·91	0·67

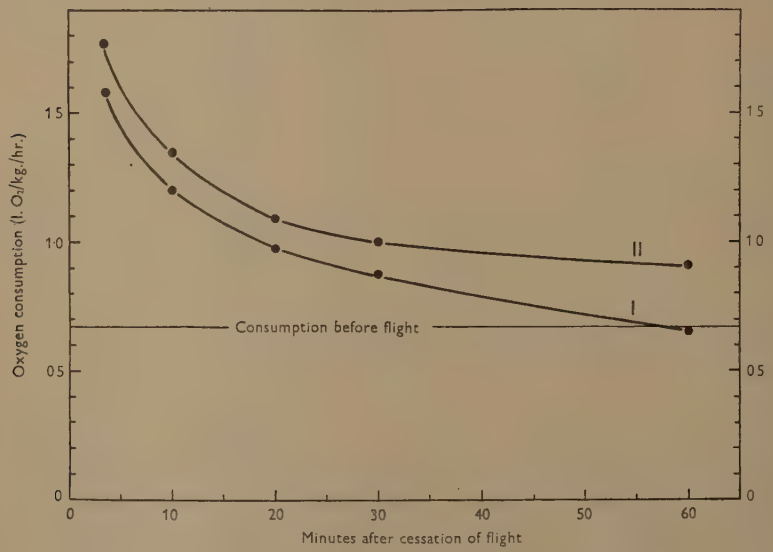


Fig. 3. Oxygen consumption and 'oxygen debt' of *Schistocerca* males after 10 (I) and 90–195 (II) min. of flight.

1 hr. In order to obtain a general expression of the increase in metabolic rate after flight, the oxygen consumption was plotted against time in the experiments in which the same animals were used during the entire period of recovery. By means of these

curves the rates could be read at arbitrary times, and Table 4 contains the figures read 3½, 10, 20, 30 and 60 min. after the cessation of flight (seven experiments).

The average consumption after 10 (I) and 90–195 min. (II) of flight respectively are shown in Fig. 3. The horizontal line at 0·67 l. O_2/kg./hr. represents the average metabolic rate before flight of the seven animals in question, and the area between this line and the curves I and II represents the total increase in oxygen uptake after flights of short and long duration respectively. According to the general terminology this amount is referred to as the 'oxygen debt' incurred during flight. It corresponded to 0·3–0·7 l. O_2/kg., and it was very characteristic that the recovery lasted at least 1 hr. even after short flights, the total 'debt' as well as the time of recovery being doubled after prolonged flight. There was a general tendency of the R.Q. to decrease during the recovery, but certainly the R.Q. could not be relied upon until the metabolism had reached a steady state, i.e. at the end of the recovery. At this state the R.Q. was low (0·7) indicating a combination of fats. On account of possible anabolic processes, however, this interpretation is dubious.

ESTIMATION OF THE ACCURACY

As far as the oxygen consumption is concerned the capacity of storing oxygen in insects which do not contain haemoglobin is determined by the amount which can be physically dissolved, and as this amount is negligible, the accuracy of the measurements of the O_2 uptake was only limited by the analytical procedure. Generally the O_2 percentage of the air in the container was lowered by 1% and the CO_2 content increased correspondingly. This means that the oxygen consumed per animal could be determined with an accuracy of about 3%, the accuracy of the gas analyses corresponding to 0·02% O_2, while the estimate of the volumes of the small containers was accurate to 1%. But when the metabolic rate per kg. body weight was concerned, the figures were only correct to about 5–7%, the weight of the individuals varying 5% from day to day according to the content of the gut.

On account of the considerable amounts of CO_2 which are reversibly combined with the blood and tissues, it is difficult to estimate the sources of error involved in the determination of the R.Q. in flying insects, and furthermore only few investigations deal with the absorption of CO_2 in insect blood. In spite of this lack of appropriate data, the low R.Q., so unexpected beforehand, of flying *Schistocerca* justifies an attempt to estimate the effect of storing or washing out of CO_2. Christensen, Krogh & Lindhard (1934) and Christensen & Hansen (1939) discussed the requirements to be fulfilled in order to obtain reliable determinations of the R.Q., especially during muscular work of man, the main requirements with simultaneous determination of the CO_2 output and the O_2 uptake being: (1) the CO_2 expired during the experiment should by far exceed the amount of CO_2 which might be washed out or stored in the body, or if this is not the case, (2) the metabolism and the ventilation should have reached at a steady state.

During the first minutes of flight the second requirement was certainly not fulfilled, and CO_2 might be washed out both due to a relatively increased ventilation, the raised body temperature, and the eventual decrease in the pH of the blood. No CO_2 dissociation curves of locusts are available and the only curves known to us refer to the blood of the honey-bee larva (Bishop, 1923) and the *Gastrophilus* larva (Levenbook, 1950). According to these curves the CO_2 capacity is about half of the capacity of human blood. The figures given by Florkin (1937) indicated the same order of magnitude in some other insects. Liljestrand (1916) found that by maximum hyperventilation during rest 2–3 l. CO_2 could

be washed out of the human body, and during heavy muscular work Christensen & Hansen (1939) demonstrated a decrease in the CO_2 content of the blood amounting to 25 vol. %. This corresponds to 1 l. CO_2 washed out from the blood alone. According to the figures recorded by Liljestrand (1916) the maximum amount liberated from the body in excess of the amount produced by the combustion would then be 5 l. CO_2, the relation between the amount of CO_2 in the blood and in the remaining tissues being about 1:4. In other words, during muscular work in man the relatively increased ventilation, the increased body temperature, and the formation of lactic acid can cause 5 l. CO_2 to be washed out of the body. This figure corresponds to 70 ml. CO_2/kg. body weight. The minimum amount of CO_2 produced by flying *Schistocerca* was 8000 ml./kg./hr., and since no experiment lasted less than 10 min. the minimum amount produced during an experiment was 1300 ml. CO_2/kg. If we adopt the above figure from human beings, the amount of CO_2 expired in excess of the amount produced would not exceed $\frac{70 \times 100}{1300}\% = 5\%$ during the first 10 min. of flight. Since as a rule, the experiments lasted longer (one and a half to three times), and the metabolic rate was higher (one and a half to three times), the error would hardly exceed 2–3%. This accuracy was sufficient, the gas analysis allowing the R.Q. to be determined with an accuracy of 2% only. In *Gastrophilus* Levenbook (1950) found that, contrary to man, the larval tissues contained similar amounts of CO_2 per unit volume as did the blood. If this is the case in locusts, too, more than 70 ml. might be washed out or stored. However, the dissociation curves show that if the CO_2 percentage in the tracheal system was altered by 1·5% only 60–70 ml. CO_2/kg. would be deliberated or stored. In the above calculation, this means that the lower CO_2 capacity of insects would compensate the effect of the relatively higher CO_2 content of the tissues; and, in fact, the measurements of the R.Q. during the first period of flight did not differ significantly from the values obtained immediately after (Table 2, Exps. 7–11).

During the successive periods of flight the animals were to be considered in a steady state and thus the gas analysis alone determined the accuracy. The same holds true in resting animals when the large containers were used, the liberated CO_2 corresponding to 1000–1500 ml. CO_2/kg./experiment. When the small containers were used, however, analogous calculations gave a much lower accuracy, but since the average R.Q. before flight was 0·82 in both series, the animals were offered the same food, and the standard deviation was only 0·06 in twenty-three experiments with the small containers, it was concluded that the locusts were in a steady state during the experiments, and so it is reasonable to consider the values of the R.Q. before and during flight a valid expression of the proportion between the CO_2 produced and the O_2 simultaneously consumed, and thus an indicator of the materials combusted. But during the initial phases of recovery the rapid changes in metabolic rate and ventilation made the results less reliable.

DISCUSSION

Oxygen consumption and oxygen debt

The diagram, Fig. 4, illustrates the changes in oxygen consumption when a locust started to perform flight movements at a moderate intensity (15 l. O_2/kg./hr.) and flew for 10 min. only. Nevertheless, the diagram gives an impression of the enormous change in the metabolic rate at the transition from rest to work, and when it is remembered that the intensity during flight might be twice as big as shown in Fig. 4, and that flying might be continued for several hours, it becomes clear that the call for oxygen and nutrients are of quite another order of magnitude in a flying than in

a resting insect. The average increase in oxygen consumption was twenty-five times the value during rest, but sometimes the metabolic rate increased fifty times. The figures do not allow an estimate of the average consumption of migrating locusts. The wing-beat frequency of a locust flying in front of a wind tunnel under rather natural conditions only differed slightly from the frequency measured when the same locust flew in the container, and so it is reasonable to think that the metabolic rates of migrating locusts do not differ essentially from the rates measured in the container. In any case, every physiological mechanism of importance during flight, like the mechanisms of ventilation, combustion, fuel mobilization, heat regulation, and so on, must be demonstrated to work at a sufficient intensity at the highest metabolic rates measured so far.

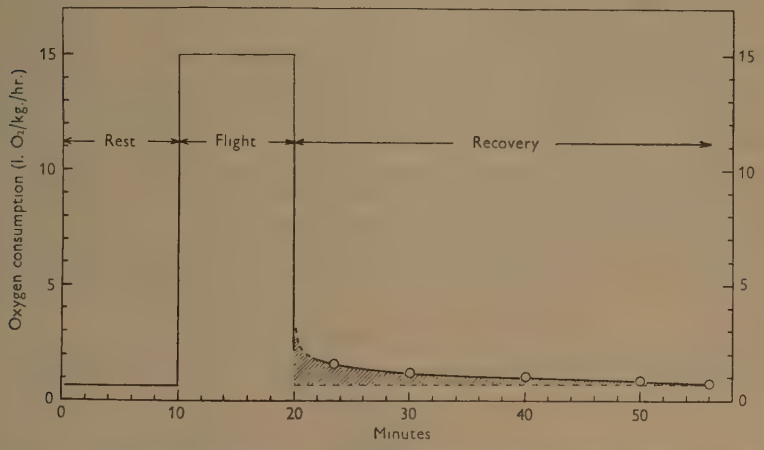


Fig. 4. Diagram. Oxygen consumption of *Schistocerca* males before, during, and after 10 min. of flight at moderate intensity. The hatched area represents the 'oxygen debt'.

The increase in metabolic rate after flight which characterized the phase of recovery amounted to 0·3–0·7 l. O_2/kg. in total. This 'oxygen debt' corresponded to the consumption during 0·5–1·5 min. of flight, only, and so anaerobic breakdown of nutrients during flight could not have amounted to much. The most conspicuous feature, however, was the considerable time, 1–2 hr., necessary to abolish the 'debt'. At the end of the recovery after even a short period of flight the R.Q. was always low (0·7), but this might be due to anabolic as well as to katabolic processes. In a later paper the supply of oxygen to the wing muscles will be dealt with: the ventilatory mechanism seemed to warrant that the 'oxygen debt' was due to other causes than lack in oxygen.

Comparison with other insects

An oxygen consumption of 10–30 l. O_2/kg./hr. in flying *Schistocerca* is considerable compared with the maximum uptake of 4 l. O_2/kg./hr. in man (Nielsen & Hansen, 1937), but the figures are of a moderate size compared with what has been found in

bees, flies and butterflies (cf. p. 344), and even in small mammals like mice and shrews metabolic rates of 20 l. O_2/kg./hr. have been recorded (Krogh, 1941; Pearson, 1947). However, the metabolism of flying insects has been studied most thoroughly in *Drosophila*, and therefore it is of special interest to compare the respiratory exchange of these small flies with the exchange of the much larger locusts. In spite of the great difference in body weight (1 : 1000) and the considerable differences in the structure of the wing apparatus, *D. repleta* consumes oxygen at a rate corresponding to 21 l. O_2/kg./hr. during tethered flight (Chadwick & Gilmour, 1940), i.e. at the same relative rate as a *Schistocerca* flying under similar conditions. But apart from the metabolic rate the metabolism during flight of these two types of insects differed considerably. Flying *Drosophila* exclusively utilized carbohydrates, whereas in flying *Schistocerca* the low values of the R.Q. showed that carbohydrates did not serve as the only source of energy in any of the experiments and, on the contrary, the lowering of the R.Q. as the flight continued indicated that the main source of energy during prolonged flight was fat. If we assume that the combustion of protein was negligible during flight compared with the combustion of fat and carbohydrates, the share of fats as a source of energy can be calculated. This assumption is reasonable, since in mammals the combustion of protein is practically independent of muscular work. The average R.Q. after more than 30 min. of flight was 0·75 only, which means that about 85% of the energy should derive from fats. It might be due to the difference in the fuels applied by the two insects that the 'oxygen debt' in *Schistocerca* was not abolished until 1–2 hr. after the flight had ceased, whereas the metabolic rate in *Drosophila* reached the resting level in less than 2 min. after the cessation of flight movements. Perhaps the elimination of ketone bodies resulting from the intensive breakdown of fats caused this prolonged recovery which was absent in *Drosophila*.

SUMMARY

The respiratory exchange of mature males of the Desert Locust *Schistocerca gregaria* (Forskål) has been studied during tethered flight in a small container and compared with the exchange before and after flights of varying duration. All determinations were based on gas analyses, and so the CO_2 output and the O_2 uptake were determined simultaneously. The accuracy of the analytical procedure has been discussed and the determinations of the R.Q. before and during flight found to be valid. The figures of the oxygen consumption are reduced to N.T.P.

1. During rest at 27–30° C. the oxygen consumption amounted to 0·63 l. O_2/kg./hr., which means that an average male of 1·8 g. consumed 18 cu.mm. O_2 per min. During flight, however, the consumption increased fifteen to fifty times, the corresponding figures being 10–30 l. O_2/kg./hr. or 300–900 cu.mm. O_2 per min. in an average male.

2. After even a short period (10 min.) of flight in a roundabout or in front of a wind tunnel a distinct 'oxygen debt' was demonstrated. After prolonged flight (90-195 min.) the 'debt' was nearly doubled. It corresponded to 0·3–0·7 l. O_2/kg. or to the oxygen consumption during only 0·5–1·5 min. of flight, so that anaerobic

processes could not amount to much. It was characteristic that the recovery lasted at least 1 hr.

3. The R.Q. in resting animals averaged 0·82, and during the first 30 min. of flight the same value was obtained, but during the following 60 min. a statistically significant decrease of the R.Q. could be demonstrated, the average value in this period being 0·75. This unexpected result strongly indicates that, unlike other insects investigated so far, locusts utilize mainly fat as a source of energy during sustained flight.

4. It is suggested that a surplus of ketone bodies caused by the intensive breakdown of fats might explain the prolonged recovery of 1–2 hr. duration in *Schistocerca*, the recovery after flight in *Drosophila* which fly on carbohydrate lasting only 2 min. or less in spite of the same relative rate of combustion.

This work is a part of an investigation on insect flight which was started by the late Prof. August Krogh, F.M.R.S. We are indebted to the Scandinavian Insulin Foundation (*Nordisk Insulinfond*) and to the Carlsberg Foundation (*Carlsbergfondet*) for financial support. We also thank Dr F. Buchthal who has undertaken the leadership of the laboratory after the death of August Krogh in September 1949. A grant from the Anti-Locust Research Centre, London, rendered possible the participation of the junior author, and special thanks are due to Dr B. P. Uvarov and to Dr D. L. Gunn for their help and interest. The Anti-Locust Research Centre delivered the locusts reared by Mr P. Hunter-Jones.

REFERENCES

Beutler, R. (1937). *Z. vergl. Physiol.* **24**, 71.
Bishop, G. H. (1923). *J. Biol. Chem.* **58**, 543.
Chadwick, L. E. (1939). *Physiol. Zool.* **12**, 151.
Chadwick, L. E. (1947). *Biol. Bull. Woods Hole*, **93**, 229.
Chadwick, L. E. & Gilmour, D. (1940). *Physiol. Zool.* **13**, 398.
Chadwick, L. E. & Williams, C. M. (1949). *Biol. Bull. Woods Hole*, **97**, 115.
Christensen, E. H. & Hansen, O. (1939). *Skand. Arch. Physiol.* **81**, 137.
Christensen, E. H., Krogh, A. & Lindhard, J. (1934). *Quart. Bull. Hlth Org. L. o. N.* **3**, 388.
Davis, R. A. & Fraenkel, G. (1940). *J. Exp. Biol.* **17**, 402.
Florkin, M. (1937). *Arch. Int. Physiol.* **45**, 241.
Fraenkel, G. (1932). *Z. vergl. Physiol.* **16**, 371.
Jongbloed, J. & Wiersma, C. A. G. (1935). *Z. vergl. Physiol.* **21**, 519.
Krogh, A. (1904). *Skand. Arch. Physiol.* **15**, 328.
Krogh, A. (1920). *Biochem. J.* **14**, 267.
Krogh, A. (1941). *The Comparative Physiology of Respiratory Mechanisms*, p. 3. Philadelphia: University Pennsylvania Press.
Krogh, A. & Weis-Fogh, T. (1951). Under preparation.
Levenbook, L. (1950). *J. Exp. Biol.* **27**, 158.
Liljestrand, G. (1916). *Skand. Arch. Physiol.* **33**, 153.
Nielsen, M. & Hansen, O. (1937). *Skand. Arch. Physiol.* **76**, 37.
Pearson, O. P. (1947). *Ecology*, **28**, 127.
Weis-Fogh, T. (1949). *Nature, Lond.*, **164**, 873.
Weis-Fogh, T. (1950). *8th Int. Congress Entom. Proc.* p. 584.
Wigglesworth, V. B. (1949). *J. Exp. Biol.* **26**, 150.
Williams, C. M., Barness, L. A. & Sawyer, W. H. (1943). *Biol. Bull. Woods Hole*, **84**, 263.

OBSERVATIONS ON THE DISTRIBUTION OF INORGANIC PHOSPHORUS, SOLUBLE CALCIUM AND SOLUBLE MAGNESIUM IN THE STOMACH OF THE SHEEP

By G. A. GARTON

From the Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 10 *November* 1950)

In a preliminary survey of the distribution and fate of phosphorus-containing compounds in the alimentary tract of the sheep it was decided to estimate the inorganic phosphate levels of the fluid of the various divisions of the stomach. Information on this topic is very scanty, the only figure for phosphorus in sheep rumen liquor which could be found in the literature being 87 mg. P/100 ml. (Brünnich & Winks, 1931). The initial experiments showed that inorganic phosphate occurred in higher concentration in abomasal liquor than was anticipated, and this led to an examination of the levels of soluble calcium and magnesium. Calcium and magnesium were chosen because their fate in the alimentary tract is often closely linked with that of inorganic phosphate. Some observations on the composition of sheep gastric juice were also made as one of the animals available for this investigation had an abomasal pouch of the Hollander type.

EXPERIMENTAL

Animals

(*a*) *Fistulated sheep.* The animals used were two Cheviot wethers (sheep nos. 1 and 2) and a Cheviot ewe (sheep no. 3). Animals nos. 1 and 2 were fitted with permanent ruminal and duodenal fistulae, the latter being immediately caudal to the pylorus. Sheep no. 3 had permanent fistulae in the rumen and abomasum, together with an abomasal pouch. The author wishes to thank Dr A. T. Phillipson who carried out the surgical operations on these animals and for allowing him to use sheep no. 3 in this investigation.

(*b*) *Slaughtered sheep.* All were adult animals; nos. 4–7 were Cheviot ewes, nos. 8 and 9 (kindly placed at the author's disposal by Dr J. Duckworth) were cross-Border Leicester ewes and no. 10 was a Suffolk cross-ewe.

Diet

(*a*) *Fistulated sheep.* Sheep nos. 1 and 2 received 450 g. chopped meadow hay twice a day (8 a.m. and 8 p.m.). For two 2 hr. periods each day the animals had access to water. They consumed all the food and drank about 1·75 l. of water per day. Sheep no. 3 received 450 g. chopped meadow hay twice a day (8 a.m. and

8 p.m.) and 225 g. of a 2:1 mixture of linseed oil cake and oats at 11 a.m. each day. The animal had access to water all day. All three sheep were housed indoors in individual pens and had access to salt and mineral licks.

(*b*) *Slaughtered sheep.* The pasture-fed sheep which were slaughtered (sheep nos. 4–7) consumed a mixture of rye-grass and clover during the months of May to September. Sheep no. 8 had been receiving an almost calcium-free diet, consisting mainly of oats, chopped oat straw, ground maize and blood meal. Sheep no. 9 received the same diet with the addition of 2·5 g. $CaCO_3$ per day. Sheep no. 10 consumed 1000 g. chopped meadow hay per day and had free access to water and a mineral lick.

Sampling

(*a*) *Fistulated sheep.* Ruminal samples (usually about 50 ml.) were withdrawn by inserting a glass tube into the fistula and removing the contents by suction. Although the ruminal samples cannot be considered as representative of the total ruminal contents the method of sampling consistently gave material of a similar composition in respect of the percentage of dry matter. Duodenal and abomasal samples (usually about 50 ml.) were obtained by withdrawing the gauze plug in the fistula and allowing the contents to flow out; the duodenal material is representative of the material leaving the abomasum (Phillipson, 1950). Samples of gastric juice were collected from the abomasal pouch of sheep no. 3 by attaching a rubber tube to the glass cannula and allowing the secretion to drain into a suitable receiver. The rate of flow of the juice varied (see Phillipson, Green, Reid & Vowles, 1949), but was usually about 50 ml./hr.

(*b*) *Slaughtered sheep.* The animals were slaughtered at 10 a.m., which in the case of sheep nos. 8 and 9 was 12 hr. after the last feed and 2 hr. in the case of sheep no. 10. As soon as possible after slaughter the various divisions of the stomach were ligated. The contents of each division were then removed, weighed, and representative samples taken for analysis.

Analytical methods

All the reagents used were of A.R. quality (British Drug Houses Ltd.). For the estimation of inorganic phosphate, soluble calcium and magnesium the samples of ruminal, abomasal and duodenal contents were strained through three thicknesses of surgical gauze to yield the corresponding liquors. Omasal samples were found to be too dry to be strained, and so a representative sample of the total contents was wrapped in surgical gauze and squeezed in a hand-press to obtain liquor. The brown-green liquor obtained by straining or squeezing was then treated as follows:

The liquor (5 ml.) was diluted with distilled water (5 ml.) and the diluate centrifuged in the superspeed head of an M.S.E. centrifuge (20,000 × *g*) for 20 min. The supernatant liquid was then pipetted off and re-centrifuged at the same speed for 10 min. The resulting supernatant liquid was clear and bacteria-free.

Inorganic phosphate determination

The clear liquid (0·5 ml.) was diluted to 25 ml. and suitable aliquots taken for the estimation of phosphorus according to Berenblum & Chain (1938). The original colour of the liquors was diluted to such an extent that it did not interfere with the determination.

Soluble calcium and magnesium determination

Duplicate analyses on all samples were carried out. The clear liquid (2 ml.) was pipetted into a 15 ml. conical centrifuge tube and saturated ammonium oxalate solution (1 ml.) added, followed by thymol blue indicator (5 drops). Dilute trichloroacetic acid (10%, w/v) was then added dropwise until the solution turned pink. Dilute ammonia (10%, v/v) was carefully added dropwise until the indicator changed to yellow, thus bringing the mixture to pH 3 at which calcium is quantitatively precipitated as oxalate uncontaminated with phosphate (see Washburn & Shear, 1932; Holth, 1949). The mixture was allowed to stand overnight and then centrifuged at 2000 r.p.m. for 10 min. The supernatant liquid was carefully decanted into a 15 ml. centrifuge tube and reserved for the determination of magnesium (see below). The precipitated calcium oxalate was washed with 2×5 ml. of dilute ammonia (2%, v/v) in the centrifuge, dissolved in N-H_2SO_4 (2 ml.) and the solution titrated against N/100 $KMnO_4$ in the usual way.

Magnesium was determined by adding 2% (w/v) diammonium hydrogen phosphate (1 ml.), followed by 7 drops of ammonia (sp.gr. 0·88) and then scratching the sides of the tube with a glass rod to facilitate crystallization of the magnesium salt. The mixture was left overnight and centrifuged at 3000 r.p.m. for 10 min. The precipitate was washed twice in the centrifuge with 5 ml. of ethanolic ammonia (Briggs, 1922) and then dissolved in N-H_2SO_4 (2 ml.). The solution was diluted to 25 ml. and suitable aliquots taken for *P* determination by the method of Berenblum and Chain (1938). $\text{Magnesium} = \text{phosphorus} \times \frac{24 \cdot 3}{31}$.

Inorganic phosphate, soluble calcium and magnesium in gastric juice were determined by similar methods after removal of the small amount of protein present by precipitation with an equal volume of 10% (w/v) trichloroacetic acid.

Dry-weight determinations

Representative samples of unstrained organ contents were dried to constant weight at 105°.

pH *determinations*

These determinations were made with a glass electrode on the liquors after straining or squeezing as soon as possible after removal of the sample from the animal.

RESULTS

The initial experiments showed that the inorganic phosphate concentration of the ruminal and duodenal contents of sheep nos. 1 and 2 was of the order of 30–40 mg. P %, expressed on the strained liquors. The inorganic phosphate level did not

depend on the time after feeding that the samples were taken. Typical results are shown in Table 1.

Since the concentration of inorganic phosphate in the material leaving the abomasum was of the same order as that found in rumen liquor in spite of the diluting effect of gastric juice, it was decided to investigate the levels of soluble calcium and magnesium as well as inorganic phosphate in the two liquors to see if a similar 'concentration effect' was apparent. The results are shown in Table 2, in which is also recorded the pH and percentage dry matter of several samples. The ruminal and duodenal or abomasal samples were withdrawn at the same time.

Food material entering the abomasum is subjected to dilution with gastric juice and so samples from the abomasal pouch of sheep no. 3 were examined. The pH was found to be usually between 1·0 and 2·0, the inorganic phosphate content 0·1–0·5 mg.%, magnesium 0·7–1·7 mg.% and calcium about 1 mg.%.

Samples of total omasal contents cannot be obtained from a living animal even if fistulation were possible, the contents between the laminae are too solid and inaccessible to allow the withdrawal of a representative sample. Consequently, analyses of omasal contents had to be performed on slaughtered animals. The analyses of the organ contents of these animals are given in Table 3. In all cases the term 'ruminal contents' includes reticulum contents, since early experiments showed no significant difference in the concentration of inorganic phosphate in these loosely separated divisions of the stomach.

Table 1. *The inorganic phosphate content of ruminal and duodenal liquors*

(The numbers in brackets refer to the number of determinations (on different days) of which the figures quoted for phosphorus are the average.)

Sheep no.	Hours since last hay feed	Inorganic P mg.%	
		Ruminal liquor	Duodenal liquor
1	2	32 (4)	35 (1)
1	5	34 (1)	—
1	8	34 (2)	32 (1)
1	12	32 (1)	32 (2)
2	2	32 (6)	37 (3)
2	5	29 (1)	—
2	8	36 (2)	32 (1)
2	12	37 (1)	36 (2)

Effect of gastric juice on ruminal contents

Samples were obtained from sheep no. 2 of ruminal contents (500 ml.) and duodenal contents (50 ml.), 4 hr. after the last hay feed. Gastric juice was collected from sheep no. 3 and centrifuged to remove a little mucus. The ruminal sample was strained through open-mesh gauze to remove large hay particles and so facilitate the measuring of volumes. The strained material is subsequently referred to as ruminal contents, since the straining only reduced the percentage of dry matter from 3·4 to 2·9%. The pH of the ruminal contents, duodenal contents and gastric juice was determined and inorganic phosphate, soluble calcium and magnesium estimated as already described, with the results shown in Table 4.

Table 2. *The inorganic phosphate, soluble calcium and magnesium content of the sheep's stomach*

(The duodenal samples from sheep nos. 1 and 2 are referred to as abomasal samples, since they are representative of the material leaving the true stomach.)

Sheep no.	Time (in hours) after last hay feed	pH of strained liquor		Inorganic phosphate (P mg. %)		Soluble magnesium (mg. %)		Soluble calcium (mg. %)		Dry matter %	
		Rumen	Abomasum	Ruminal liquor	Abomasal liquor	Ruminal liquor	Abomasal liquor	Ruminal liquor	Abomasal liquor	Ruminal sample	Abomasal sample
1	2	—	—	35	48	11	11	13	42	—	—
1	2	5·9	3·3	32	42	13	13	17	40	4·3	4·3
1	6	6·5	3·0	31	42	11	14	15	44	4·1	5·3
1	7	5·75	3·2	32	42	13	16	17	53	3·3	4·9
1	12	6·55	3·4	37	43	9	12	11	53	5·2	4·9
2	2	5·9	3·4	33	43	13	13	14	38	—	—
2	2	—	—	38	39	12	13	—	—	—	—
2	2	5·8	3·05	30	34	13	13	21	42	3·5	4·0
2	2	6·35	3·6	28	39	12	13	18	45	3·0	3·7
2	6	6·4	2·8	35	38	11	12	15	49	4·4	4·3
2	7	5·85	3·0	27	36	10	14	13	53	3·9	4·6
2	12	6·6	3·1	41	43	8	12	11	50	6·3	5·7
2	12	6·4	3·1	39	49	7	12	11	56	5·5	4·7
3	3	—	—	72	72	14	19	23	43	—	—
3	3	—	—	73	93	15	20	19	39	—	—
3	3	—	—	80	92	20	23	20	40	—	—
3	3	—	—	81	100	11	18	10	65	—	—
3	3	—	—	65	90	13	23	10	67	4·1	7·7
3	3	—	—	70	73	—	—	—	—	4·3	8·2
3	3	6·5	3·2	60	75	13	21	11	67	4·5	—

Table 3. *Analysis of the stomach contents of slaughtered sheep*

(R = ruminal liquor, O = omasal liquor, A = abomasal liquor.)

Sheep no.	pH of strained liquor			Inorganic phosphate (P mg.%)			Soluble magnesium (mg.%)			Soluble calcium (mg.%)			Dry matter (%) (the figures in brackets give total weight (g.) of organ contents)			Diet
	R	O	A	R	O	A	R	O	A	R	O	A	Ruminal contents	Omasal contents	Abomasal contents	
4	—	—	—	42	—	42	—	—	—	—	—	—	10·9 (8580)	— (23)	7·8 (518)	Rye grass and clover
5	—	—	—	63	71	53	—	—	—	—	—	—	10·4 (5050)	20·7 (61)	5·2 (384)	Rye grass and clover
6	6·6	6·6	6·2	66	99	52	6	9	7	8	9	14	9·8 (5330)	19·1 (90)	5·9 (242)	Rye grass and clover
7	6·5	6·5	6·2	68	92	56	12	13	9	8	10	13	10·9 (6540)	17·3 (41)	7·0 (158)	Rye grass and clover
8	6·1	5·9	3·3	164	212	102	5	7	9	0·5	1·5	8	16·2 (—)	20·6 (58)	11·5 (1037)	Oats, chopped oat straw, ground maize and blood meal
9	6·1	5·8	3·4	117	207	99	2	5	8	0·5	1·5	13	11·7 (—)	22·2 (128)	10·9 (511)	Oats, chopped oat straw, ground maize and blood meal +2·5 g. $CaCO_3$/day
10	6·4	6·4	4·7	35	37	36	10	12	8	18	19	31	11·7 (14800)	15·0 (326)	7·5 (970)	Chopped meadow hay

To simulate the acidity of duodenal (abomasal) contents a mixture of the gastric juice (65 ml.) and the ruminal contents (25 ml.) was made. The mixture (pH 2·65) was sampled immediately and after 3 hr. at 39°. In Table 4 are recorded the results of the analyses of these samples for inorganic phosphate, soluble calcium and magnesium.

Table 4. *Analysis of ruminal and duodenal contents of sheep no. 2, gastric juice of sheep no. 3 and a mixture of ruminal contents (25 ml.) and gastric juice (65 ml.)*

Sample	pH	Inorg. P. mg.%	Soluble Ca mg.%	Soluble Mg mg.%
Gastric juice	1·2	0·2	1	0·7
Duodenal contents	2·7	37	35	8
Ruminal contents	6·4	35	15	7
Ruminal contents and gastric juice on mixing	2·65	10 (35·5)*	9·4 (31·2)*	2·8 (8·3)*
Ruminal contents and gastric juice after 3 hr. at 39°	—	10 (35·5)*	9·1 (30·2)*	2·8 (8·3)*

* The figures in brackets give the results expressed on ruminal contents after allowing for the amounts of the respective ions present in the gastric juice of the mixture.

Effect of synthetic saliva and gastric juice on hay

Since the experiments already described show a considerable release of calcium by gastric juice from the ruminal contents of hay-fed sheep, the effect of synthetic saliva and gastric juice on hay was studied. The sample of hay used was obtained from the bulk store used for feeding sheep nos. 1–3.

A solution of salts resembling in composition that of sheep saliva was prepared essentially according to Elsden (1946), as modified by Oxford (1951) and gastric juice was collected from sheep no. 3. The synthetic saliva was allowed to stand overnight at room temperature, after which it had a pH of 6·85.

Finely chopped hay (10 g.) was placed in a 250 ml. conical flask and synthetic saliva (100 ml.) then added. The flask was shaken and the pH of the mixture determined. It was then loosely stoppered and put into the incubator at 39° along with another flask containing only synthetic saliva (100 ml.). The flasks were shaken every half hour and removed from the incubator after 5 hr. The pH of the contents of the flasks was determined and the mixture containing hay was filtered. Inorganic phosphate, soluble calcium and magnesium were determined on the filtrate and on the sample of synthetic saliva which had been similarly incubated.

The experiment using gastric juice was carried out in a similar manner using hay (10 g.) and a mixture of gastric juice (75 ml.) and distilled water (25 ml.) in order to simulate conditions in the abomasum. The second (control) flask in this case contained 100 ml. of the same diluted gastric juice. The results of these experiments are given in Table 5.

Table 5. *The effect of synthetic saliva and gastric juice on hay at 39° for 5 hr.*

Sample	Initial pH	Final pH	Inorganic P (mg. % on liquid)	Soluble Ca (mg. % on liquid)	Soluble Mg (mg. % on liquid)
Synthetic saliva	6·85	7·9	23	1·5	2·7
Synthetic saliva extract of hay*	6·85	7·7	14	22	16·2
Diluted gastric juice	1·5	1·5	0·1	1·1	0·8
Diluted gastric juice extract of hay	1·5	3·3	10	59	19·4

* A small amount of crystalline material separated from this extract and was found to contain calcium and phosphate.

DISCUSSION

The inorganic phosphate concentration in the fluid of the rumen and abomasum of the sheep appears to be fairly constant on a given diet. The rumen of the sheep has no secretory glands of its own and the saliva provides much of the liquid medium of ruminal contents. McDougall (1948), in a study of sheep saliva, found that the secretion of parotid saliva (the major component of mixed saliva) ranged from 2 to 4 l. per day. Phosphorus in mixed saliva was found to be entirely in the inorganic form (37–72 mg. P%), calcium, 1·6–3·0 mg.% (considered to be largely ionized) and magnesium 0·6–1·0 mg.%.

It is clear that ruminal fluid is a good buffer in which phosphate, along with bicarbonate, plays an important role in buffering acid production resulting from bacterial fermentation. Van der Wath (1942) showed that the bacterial population of the sheep's rumen was increased by the addition of phosphate to a diet low in phosphorus. The phosphate in the rumen doubtless plays an important part in the fermentation reactions themselves, most of which probably involve phosphorylation mechanisms.

The inorganic phosphate content of the ruminal liquor of sheep fed exclusively on hay (nos. 1, 2 and 10) or on rye-grass and clover (nos. 4–7) is of the same order as that given by McDougall (1948) for saliva, and it would seem that very little soluble phosphate is derived from such feeds in the rumen. If, however, the diet contains linseed oil cake and oats (sheep no. 3) or oats and ground maize (sheep nos. 8 and 9), the inorganic phosphate content of the ruminal liquor is considerably raised above the level found in hay- or pasture-fed animals. It seems likely that this extra phosphate is derived from the phytates present in the diet, for Reid, Franklin & Hallsworth (1947) have shown that phytates are almost completely hydrolysed quite rapidly in the sheep's rumen, probably under the influence of enzymes of micro-organisms.

On the other hand, the soluble calcium and magnesium values found for the ruminal liquor of hay-fed sheep are higher than those reported for saliva and can

be accounted for by the simple solution of these ions from the food. This has been demonstrated *in vitro* (Table 5) which shows that 20·5 mg. of calcium and 13·5 mg. of magnesium pass into solution from 10 g. of hay in 5 hr. at 39° in 100 ml. synthetic saliva over a pH range of 6·85–7·7. An interesting point in connexion with the results presented in Table 5 is that the free inorganic phosphate found in the synthetic saliva extract of hay was less than that originally present in the synthetic saliva itself. The pH of the extract rose from 6·85 to 7·7, resulting in the precipitation of a little of the phosphate, probably as calcium phosphate. This might occur *in vivo* if rumen contents ever become alkaline.

The work of Phillipson *et al.* (1949) has shown that the dilution by gastric juice of food matter leaving the sheep's omasum is quite considerable, being of the order of 1–2 parts of gastric juice to 1 part of omasal material. Since gastric juice has been shown to contain very little phosphate, calcium and magnesium, an explanation must be sought to account for the maintenance of the concentration of inorganic phosphate and soluble magnesium in abomasal contents, and also to account for the greatly increased amounts of soluble calcium found in the abomasum. It could therefore be that there is a liberation of hitherto insoluble phosphate, calcium and magnesium in the abomasum under the influence of gastric juice or that absorption of water occurs in the omasum, resulting in the passage of a concentrate of ruminal and reticulum contents into the abomasum. The results presented in Table 4 show that gastric juice does not effect any significant release of phosphate from the rumen contents of a hay-fed sheep, though magnesium is liberated to a slight extent. Calcium, on the other hand, undoubtedly passes into solution in considerable amounts as soon as gastric juice and ruminal contents are mixed, or when hay is incubated with diluted gastric juice (Table 5).

For many years the omasum, with its highly specialized structure has attracted the attention of physiologists, giving rise to much speculation regarding its function in the ruminant. Favilli (1937) reviewed much of the data on this subject and concluded that the omasum does not function merely to press and squeeze the food, but also to effect comminution and to absorb water. The work on omasal contraction has been summarized by Dukes (1947, p. 315), who considers that food material entering the omasum is subjected to considerable pressure and 'some of the liquid so released probably undergoes absorption'.

The percentage of dry matter in omasal contents is considerably higher than that found in ruminal or abomasal contents (see also Elsden, Hitchcock, Marshall & Phillipson, 1946; Dukes, 1947, p. 330). This is shown in Table 3, together with the figures for inorganic phosphate, soluble calcium and magnesium in omasal liquor, which lend support to the idea that water absorption occurs during the passage of food through this division of the stomach. In all cases the concentration of these three ions is greater in omasal liquor than in the corresponding ruminal liquor. The figures for sheep nos. 6–9 are particularly striking in this respect; the percentage of dry matter in the various divisions of the stomach, taken together with the inorganic phosphate concentrations of the corresponding liquors (Table 3), can be correlated with the findings of Phillipson *et al.* (1949). If the omasal material of

these animals were diluted with about one volume of gastric juice the percentage of dry matter and the phosphate concentration of the liquor would be lowered to give figures in agreement with those found in abomasal contents.

The figure of 6·2 in Table 3 for the pH of the abomasal contents of grass-fed sheep nos. 6 and 7 is rather high. No explanation of this finding can be offered, though it may be related in some way to the comparatively small total weights of the abomasal contents of these animals. Another apparently anomalous figure in Table 3 is the low value for phosphate in the omasal liquor of sheep no. 10; this is associated with a much greater weight of total omasal contents than was found in any of the other animals.

However, the main findings of the present work point to the absorption of water from the omasum of the sheep playing an important part in the water economy of the alimentary tract, water added to the food in the form of gastric juice more or less compensating for that which is absorbed as the food passes through the omasum. The concentrations of inorganic phosphate and magnesium in abomasal liquor are thus kept at about the same level as are found in the corresponding ruminal liquor.

The picture in respect of calcium is slightly different from that of inorganic phosphate and magnesium in view of the much higher percentages of soluble calcium found in abomasal liquor than were found in ruminal or omasal liquor (see Tables 2 and 3). Omasal absorption of water probably accounts for this in some measure, but, in addition, the acidity of abomasal liquor (due to the presence of gastric juice), results in an immediate liberation of calcium as is clearly shown in Table 4. The combined form of calcium from which it is released is not a phosphate or phosphates in sheep on a hay diet, since no parallel increase in inorganic phosphate occurred when ruminal contents and gastric juice were mixed. The nature of the calcium compound or compounds from which it is liberated remains to be investigated further.

SUMMARY

1. The inorganic phosphate, soluble calcium and soluble magnesium levels in the various divisions of the stomach of the sheep on different diets have been studied.

2. The concentration of inorganic phosphate and soluble magnesium in ruminal and abomasal liquors was of the same order in these two divisions of the stomachs of three fistulated animals independent of the type of feed or time after feeding at which the samples were taken.

3. The concentration of inorganic phosphate, soluble calcium and magnesium in omasal liquor of slaughtered sheep was higher in all cases than the corresponding value for rumen liquor, lending support to the idea that one of the functions of the omasum is to absorb water.

4. Although the concentration of soluble calcium in omasal liquor was higher than that found in the corresponding ruminal liquor, much higher levels were found in abomasal liquor than in omasal or ruminal liquors.

The author is indebted to many members of the staff of this Institute for helpful discussion, particularly Dr A. E. Oxford, Dr A. T. Phillipson and Mr P. J. Heald, and to Mr W. R. H. Duncan for his technical assistance.

REFERENCES

BERENBLUM, I. & CHAIN, E. (1938). Colorimetric determination of phosphate. *Biochem. J.* **32**, 295–8.
BRIGGS, A. P. (1922). A colorimetric method for the determination of small amounts of magnesium. *J. Biol. Chem.* **52**, 349–55.
BRÜNNICH, J. C. & WINKS, W. R. (1931). Nutrition of sheep. Analysis of paunch contents. *Qd agric. J.* **35**, 183–8.
DUKES, H. H. (1947). *Physiology of Domestic Animals*, 6th ed. Ithaca, N.Y.: Comstock.
ELSDEN, S. R. (1946). The fermentation of carbohydrates in the rumen of the sheep. *J. exp. Biol.* **22**, 51–61.
ELSDEN, S. R., HITCHCOCK, M. W. S., MARSHALL, R. A. & PHILLIPSON, A. T. (1946). Volatile acids in the digesta of ruminants and other animals. *J. exp. Biol.* **22**, 191–202.
FAVILLI, N. (1937). Struktur und Tätigkeit des dritten Magens (Blättermagen oder Psalter) bei den Hauswiederkäuern. *Dtsch. tierärztl. Wschr.* Nr. 37, 45. Jahrgang, pp. 592–4.
HOLTH, T. (1949). Separation of calcium from magnesium by oxalate method. A critical study. *Anal. Chem.* **21**, 1221–6.
MCDOUGALL, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* **43**, 99–108.
OXFORD, A. E. (1951). The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. *J. Gen. Miocrobiol.* **5**, 83–90.
PHILLIPSON, A. T. (1950). Private communication.
PHILLIPSON, A. T., GREEN, R., REID, R. S. & VOWLES, L. E. (1949). The passage of food through the abomasum of the sheep. *Brit. J. Nutrit.* **3**, Proc. iii–iv.
REID, R. L., FRANKLIN, M. C. & HOLDSWORTH, E. G. (1947). Utilization of phytate-phosphorus by sheep. *Aust. Vet. J.* **23**, 136–40.
VAN DER WATH, J. G. (1942). Thesis, University of Pretoria. (Cited by McDougall, 1948.)
WASHBURN, M. L. & SHEAR, M. J. (1932). Composition of bone. XIII. Direct gravimetric determination of Ca, Mg and PO_4. *J. Biol. Chem.* **99**, 21–41.

WATER UPTAKE AND MOULTING IN *BUFO REGULARIS* REUSS

By R. F. EWER

From the Department of Zoology, University of Natal

(*Received 2 January* 1951)

(With Two Text-figures)

INTRODUCTION

Jørgensen (1949) has shown that in *Bufo bufo* and *Rana temporaria* moulting is accompanied by an increased rate of water uptake through the skin. The experiments of *Bufo regularis* Reuss about to be described confirm and in some respects extend Jørgensen's findings. In this species moulting takes place very rapidly, and it is therefore easy to follow the changes in the rate of water uptake over the whole period of moulting. The old skin starting to come away from the lower surface of the legs is usually the first sign of a moult. When the animal is kept in water at 26° C. the old skin may be almost completely shed within an hour, but usually this takes 2 hr. and occasionally as much as 3 hr.

MATERIAL AND METHODS

The present work arose out of studies on the water balance of *B. regularis* which involved measurement of the water absorbed through the skin and of the urine output at hourly intervals (Ewer, 1950). Occasionally an animal moulted during the course of an experiment, and if this happened the rates of water uptake and urine production were measured at hourly intervals until the moult had been completed. All experiments were carried out at 26° C.

RESULTS

Seven moulting animals were studied. In all cases changes in the rate of water uptake were found to accompany moulting. Fig. 1 shows the results of a typical experiment; in this case moulting was rapid and was completed within an hour. The rate of water uptake rises as moulting begins, and falls rapidly immediately afterwards, reaching in 2 hr. a value which is normal for an animal of about 50 g. weight.

In order to sum the results the figures are treated as follows. The level to which the water uptake sinks after moulting is taken as 100 and the rates of uptake for the previous hourly periods are expressed as percentages of this value. These 100% values are in each case found to fall within the normal range for non-moulting toads of corresponding weight. Each set of readings is then arranged with the point at which moulting was first observed to have started as zero time, and the

other values arranged 1, 2, 3, etc. hours before or after the commencement of moulting: mean values are then calculated for each hourly period. Table 1 shows the results arranged thus. Moulting is not necessarily detected with equal ease in every case and a second method of summing the results is possible, in which the

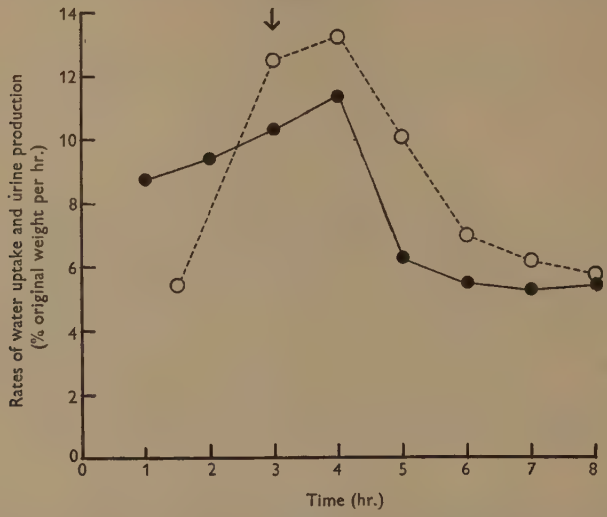


Fig. 1. Rates of water uptake and of urine production of a *Bufo regularis* of weight 54 g. Closed circles, rate of water uptake; open circles, rate of urine production. The arrow indicates the point at which moulting was first observed.

Table 1. *Relative rate of water uptake of* Bufo regularis *during moulting*

(Zero time is the point at which the first signs of moulting were observed. The figures in the last column give the rate of water uptake, expressed as percentage of original weight per hour, which has been taken as 100% in each experiment.)

Hours before or after commencement of moulting									Weight (g.)	100%
−3	−2	−1	0	+1	+2	+3	+4	+5		
—	160	171	188	206	115	100	96	99	54·1	5·5
—	—	—	234	131	116	102	115	97	50·8	5·5
—	—	293	216	173	102	94	104	—	36·9	6·0
142	156	180	181	122	99	—	—	—	63·1	4·0
—	—	173	195	158	92	100	100	—	46·0	4·5
—	122	125	143	168	120	106	104	93	42·2	4·0
—	157	151	119	120	138	96	104	—	55·7	3·7
142	149	182	168	154	112	100	104	96	Means	

time at which water uptake is at a maximum in each experiment is taken as zero time. Fig. 2 shows the results of both these methods of treating the data. The true picture probably lies somewhere between the two graphs. The first method of summing spreads the peak values too widely, since it is not possible to

determine exactly the instant at which moulting starts; while the second method takes all the peak values as simultaneous, regardless of any individual variation which may exist.

The increased rate of water uptake is not due to the new skin being more delicate and more permeable than the old, for the water uptake starts to rise before the old skin shows any signs of being shed, and falls rapidly as soon as the old skin has come away. Moulting in the Amphibia is under endocrine control, and it seems reasonable to suppose that the increased rate of water uptake is a reflexion of the

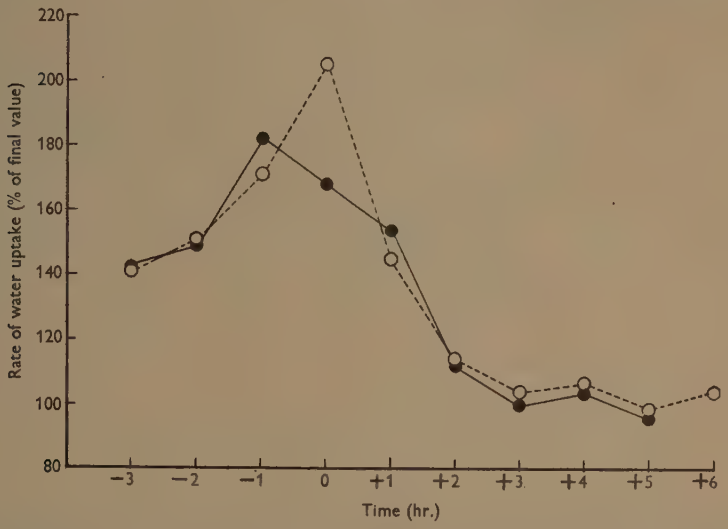


Fig. 2. Summed results of seven experiments showing the rate of water uptake in *Bufo regularis* during a moult. Closed circles, zero time taken as when moulting was first observed; open circles, zero time taken as when water uptake was maximal.

hormonal changes responsible for the initiation of moulting. In *Triturus viridescens* moulting is controlled by the thyroid, which in turn is controlled by the anterior pituitary (Adams, Richards & Kuder, 1930; Adams, Kuder & Richards, 1932). In the Anura the matter is less simple. Although the thyroid may be involved it appears to play a less important role than it does in *Triturus* (Ungar, 1933; Aubrun, 1935). Heller (1941) has shown that anterior pituitary extracts have no effect on water balance in *Rana*, while previous work on the effect of thyroxine on water balance in Anura is inconclusive (Biasotti, 1923; Heller, 1930). It thus seemed necessary to find out whether in *Bufo regularis* thyroxine increases the rate of water uptake, and experiments to test this point were therefore carried out. The experimental animals were given 0·25 mg. thyroxine by mouth on 4 successive days, and the rate of water uptake was then measured for 3 hr. periods starting 2 hr. after the last thyroxine feeding. There was no indication that thyroxine feeding

caused the animals to moult more frequently than usual, but during the course of the experiments two animals did moult. These two behaved exactly like normal moulting animals and showed the usual associated changes in water uptake. For ten animals which did not moult during the course of the experiments the mean rate of water uptake was found to be $5{\cdot}25 \pm 0{\cdot}23\%$/hr. This is not significantly different from the value of $5{\cdot}18 \pm 0{\cdot}18\%$/hr. found for normal animals of the same weight range. The increase in water uptake accompanying moulting in *B. regularis* cannot therefore be due to increased thyroid activity.

Jørgensen (1947) finds that adrenaline increases the permeability to salt of isolated frog skin, and suggests (1949) that the increased water uptake of moulting Anura may possibly be due to adrenaline. Brunn (1921) and Adolph (1935), however, found that adrenaline injection did not affect the rate of water uptake in whole animals. That posterior pituitary extracts do increase the rate of water uptake in Anura is well established, and it is therefore possible that the water balance changes accompanying moulting are a reflexion of increased posterior pituitary activity. In this connexion the values found in the present experiments for rate of urine production are of interest. Injection of mammalian posterior pituitary extract causes not only an increased water uptake, but also a marked anuresis in *B. regularis* (Ewer, 1950). In the present experiments there was no indication of any diminution of urine flow during or immediately before moulting. Fig. 1 shows the rate of urine production in a typical experiment; far from falling it actually increases considerably as moulting becomes imminent. The graph for urine production lags somewhat behind that for water uptake. As a consequence the weight of the animals when kept in water increases just before moulting and does not return to normal until after the moult has been completed. An injection of mammalian pituitrin sufficient to increase the rate of water uptake to the degree shown in Fig. 1 would have been accompanied by an almost complete anuresis lasting at least 2 hr. One might therefore assume that the absence of anuresis indicates that posterior pituitary activity cannot be the factor responsible for the observed increase in water uptake. This does not necessarily follow. Heller (1941) has shown that the anti-diuretic activity of anuran posterior pituitary extracts is very slight compared with that of mammalian extracts, and Jørgensen (1950) finds that while in some Anura extracts of the animal's own pituitary are anti-diuretic in others there is no such effect. In the present case the increased water load would tend to produce an increased urine flow, and this might be sufficient to override and mask a slight anti-diuretic effect. The absence of anuresis cannot therefore be taken as a definitive proof that posterior pituitary activity is not responsible for the increased rate of water uptake accompanying moulting. The factor responsible for this phenomenon cannot yet be identified with certainty, and further experiments on the subject are planned.

SUMMARY

1. In *Bufo regularis* Reuss moulting is accompanied by an increase in the rate of water uptake through the skin. An increase in urine flow is also observed, starting some time after the rate of water uptake has begun to increase.

2. No increase in rate of water uptake occurs following thyroxine feeding on 4 successive days.

3. The possibility that posterior pituitary activity is responsible for the observed increase in rate of water uptake during moulting is discussed.

This work was carried out during the tenure of a research grant from the South African Council for Scientific and Industrial Research.

REFERENCES

Adams, A. E., Kuder, A. & Richards, L. (1932). *J. exp. Zool.* **63**, 1.
Adams, A. E., Richards, L. & Kuder, A. (1930). *Science*, **72**, 323.
Adolph, E. F. (1935). *Amer. J. Physiol.* **115**, 200.
Aubrun, E. A. (1935). *C.R. Soc. Biol., Paris*, **120**, 504.
Biasotti, A. (1923). *C.R. Soc. Biol., Paris*, **88**, 361.
Brunn, F. (1921). *Z. ges. exp. Med.* **25**, 170.
Ewer, R. F. (1950). *J. exp. Biol.* **27**, 40.
Heller, H. (1941). *J. Physiol.* **100**, 125.
Heller, J. (1930). *Arch. exp. Path. Pharmacol.* **157**, 298.
Jørgensen, C. B. (1947). *Acta physiol. Skand.* **14**, 213.
Jørgensen, C. B. (1949). *Acta physiol. Skand.* **18**, 171.
Jørgensen, C. B. (1950). *Acta physiol. Skand.* **22**, 7.
Ungar, I. (1933). *C.R. Soc. Biol., Paris*, **112**, 504.

THE EFFECT OF PITRESSIN AND PITOCIN ON WATER BALANCE IN *BUFO REGULARIS* REUSS

By R. F. EWER

From the Department of Zoology, University of Natal

(*Received* 3 *February* 1951)

(With Four Text-figures)

I. INTRODUCTION

In Anura injection of pituitrin is followed by an increase in weight if the animals are kept in water. In a previous communication (Ewer, 1950), it was shown that in *Bufo regularis* this is the result both of an increased rate of water uptake through the skin and a decreased rate of urine elimination through the kidneys. The possibility existed that the renal and dermal components of this water-balance effect might be due to different fractions of the pituitary extract, and the desirability of investigating separately the effects of the pressor and the oxytocic fractions was pointed out. The experiments described below were carried out in order to investigate this point.

II. MATERIAL AND METHODS

The species used was *B. regularis* Reuss, and the pituitary preparations were Parke Davis pitressin and pitocin. All experiments were carried out at 26° C.

The method used was the same as that described previously (Ewer, 1950). Water uptake and urine production were measured at hourly intervals for 4 hr. before injection. The appropriate injection was then made into the crural lymph sac, and measurements were continued at hourly intervals until the animal's weight had reached a maximum. As before, the values for the first hour, when recovery from the anaesthetic may not be complete, were discarded. The mean values for the 3 hr. preceding injection give the normal rate of water uptake and urine production for the animal. The values for the post-injection period are expressed as percentages of the normal values for the same animal, and are referred to as the relative water uptake and relative urine production. In the present experiments the value for the relative water uptake in the hour following injection has been calculated, as well as the mean value for the whole period from the time of injection until the animal's weight reaches a maximum.

III. RESULTS

(1) *General*

Experiments were carried out at a series of dosages ranging from 0·1 to 25 i.u./100 g. body weight for pitressin and from 1 to 15 i.u./100 g. for pitocin. Fig. 1 shows the results of two typical experiments, one with pitressin, the other with pitocin

injection. The general picture is similar to that previously found with pituitrin injection. Injection is followed by a sharp rise in the rate of water uptake accompanied by a rapid fall in urine production; the two processes result in an increase

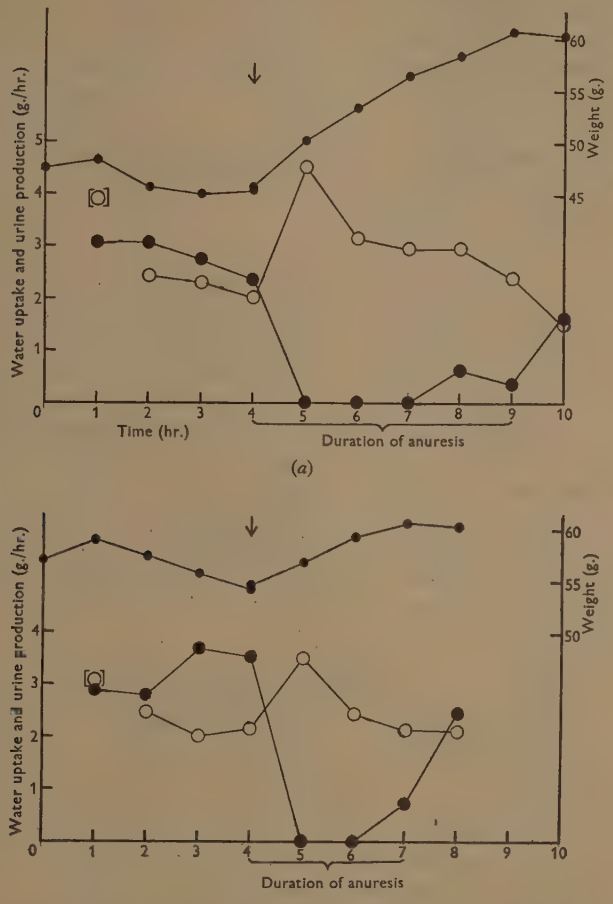


Fig. 1. (*a*) Typical experiment with pitressin injection. (*b*) Typical experiment with pitocin injection. Small filled circles, weight of toad (right-hand scale); large open circles, rate of water uptake in previous hour; large filled circles, rate of urine production in previous hour. The arrows indicate the times at which the injections were made.

in the weight of the animal. Water uptake is maximal in the hour following injection and thereafter returns gradually to normal in the course of a few hours. Urine production remains negligible, or may even cease completely, for some time, but its

recovery to normal (or to a value greater than normal) is very sudden. The period indicated in the graphs as the 'duration of anuresis' can therefore easily be determined. The point at which the effect on water uptake ceases is less definite, and the duration of this effect cannot always be determined with certainty. In calculating the mean values given in the tables any experiments in which the duration of the increased uptake is doubtful have been neglected.

The summed results for all dosages studied are given in Tables 1 and 2. It can be seen from these tables that both water uptake and urine production are affected by both extracts. It can also be seen that at the lowest dosages used, where a definite effect on water uptake is just manifested, there is already a very strongly marked anti-diuretic effect. The threshold for the renal effect is therefore presumably lower than for the dermal effect.

(2) *Effects on water uptake*

Fig. 2 shows the relative water uptake for the first hour after injection, and for the total post-injection period after pitressin and after pitocin injection. Pitressin has the greater effect. The maximum rate of uptake after pitressin injection is $231{\cdot}2 \pm 15{\cdot}64$ at a dosage of 5–10 i.u./100 g. body weight, as compared with $189{\cdot}5 \pm 11{\cdot}61$ at a dosage of 1–5 i.u./100 g. of pitocin. This difference is of doubtful significance ($P > 0{\cdot}05$), but since all the values for relative water uptake in the first hour after pitressin injection are greater than those following pitocin injection the greater effect of pitressin is clear. In addition, pitressin is effective at lower concentrations than is pitocin, giving a definite effect at 0·1 i.u./100 g., while approximately 0·5 i.u./100 g. of pitocin is required to produce a comparable effect. With both extracts the effect becomes less when very high dosages are used: this is more marked in the case of pitocin, so that pitressin is effective over a greater range of dosages than is pitocin.

Pitocin and pitressin are not complete separations of the oxytocic and pressor fractions of pituitrin: each contains some 5–10% of the other. It therefore becomes necessary to consider whether the effect of pitocin could in reality be due to its pressor fraction content. If this is so, and there are no other complicating factors, then the curves for the effects of pitressin and pitocin on water uptake should be superposable by alteration of the dosage scale. There is, however, the complication of diminishing effectiveness with increasing dosage. Since this is more marked with pitocin than with pitressin the former extract exerts a large effect over a very small range of dosages, while the latter is highly effective over a wide range, and the curves are therefore of quite different shapes. Whether the reduced response represents a true reversal of action at high dosages, or is the result of some damage to the animal, has not been investigated. Since its mechanism is not understood it is desirable in comparing the effects of pitressin and pitocin to consider only the lower dosages, where the complication of diminishing effectiveness with increasing concentration does not arise. If pitocin owes its activity to the presence of 5–10% of the pressor fraction then a dosage of 0·5–1 i.u./100 g. of pitocin should have approximately the same effect as pitressin at a dosage of 0·1 i.u./100 g.; and at

Table 1. *Effects of pitressin injection*

Dosage (i.u./100 g.)	Relative water uptake		Relative urine production	Maximum weight gain (% original)	Duration of anuresis (hr.)	Duration of increased uptake (hr.)	No. of experiments
	First hour	Total period					
0·1	130·7 ± 12·40	119·3 ± 14·90	17·2 ± 3·80	18·5 ± 2·74	2·16 ± 0·17	1·83	6
0·5–1	188·5 ± 8·19	152·5 ± 9·54	25·8 ± 5·92	19·2 ± 2·99	1·83 ± 0·40	2·16	6
1–5	197·7 ± 33·24	137·7 ± 14·36	18·3 ± 2·51	22·3 ± 5·13	2·66 ± 0·42	2·50	6
5–10	231·2 ± 15·64	148·6 ± 6·48	14·6 ± 2·91	22·5 ± 2·20	3·17 ± 0·33	3·09	12
10–15	219·7 ± 31·16	151·8 ± 16·34	17·3 ± 4·97	17·0 ± 1·57	3·00 ± 0·45	2·5	6
15–20	172·0 ± 34·25	118·2 ± 21·74	14·7 ± 5·91	18·7 ± 2·53	3·50 ± 0·43	2·0	6
20–25	156·2 ± 20·05	109·5 ± 12·52	9·3 ± 2·87	20·3 ± 2·96	3·67 ± 0·67	2·8	6

Table 2. *Effects of pitocin injection*

Dosage (i.u./100 g.)	Relative water uptake		Relative urine production	Maximum weight gain (% original)	Duration of anuresis (hr.)	Duration of increased uptake (hr.)	No. of experiments
	First hour	Total period					
0·5–1	119·5 ± 8·97	102·66 ± 7·15	51·0 ± 10·30	5·5 ± 0·2	1·1 ± 0·17	0·8	6
1–5	189·5 ± 11·61	147·3 ± 11·96	46·0 ± 13·22	10·0 ± 1·02	1·5 ± 0·43	3·0	6
5–10	173·0 ± 15·96	142·7 ± 16·46	28·4 ± 6·07	14·3 ± 2·29	1·9 ± 0·26	2·6	7
10–15	148·5 ± 16·39	121·3 ± 11·97	42·5 ± 8·42	9·0 ± 1·38	1·3 ± 0·40	1·8	6

5–10 i.u./100 g. its effect should be similar to that of pitressin at 0·5–1 i.u./100 g. The figures are as follows:

Pitocin: 0·5–1 i.u.; 1st hour 119·5 ± 8·97; total period 102·66 ± 7·15
Pitressin: 0·1 i.u.; 1st hour 130·7 ± 12·40; total period 119·3 ± 14·90

Pitocin: 5–10 i.u.; 1st hour 173·0 ± 15·96; total period 142·7 ± 16·46
Pitressin: 0·5–1 i.u.; 1st hour 188·5 ± 8·19; total period 152·5 ± 9·54

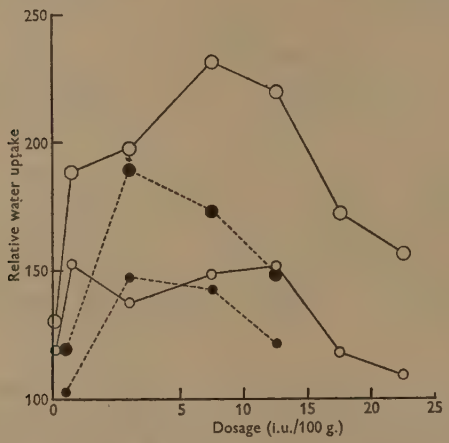


Fig. 2. (Note: in Figs. 2–4 the horizontal scale from 0–5 i.u. has, for convenience, been doubled.) Relative water uptake after injection of various dosages of pitressin and pitocin. Large open circles, first hour after pitressin injection; large filled circles and broken line, first hour after pitocin injection; small open circles, total period after pitressin injection; small filled circles and broken line, total period after pitocin injection.

In no case is the difference between the effects of the two extracts significant. It therefore appears that as far as its effect on water uptake is concerned the activity of pitocin may be attributed to its content of the pressor fraction.

(3) *Effects on urine production*

Fig. 3 shows the relative urine production and the duration of anuresis after pitressin and after pitocin injection. The effect of pitressin is much greater than that of pitocin, both in intensity and in duration. These differences are significant. For both extracts for the range from 0·5 to 15 i.u./100 g. the responses seem to be almost independent of the dosage. Analysing variance for relative urine production over this range we find that, as expected, dosage has little effect ($F3/3 = 4·3$), whereas the difference between the effects of pitressin and pitocin is highly significant ($F1/3 = 46·0$). Similarly, in the case of duration of anuresis the effect is again almost constant over the range of dosages considered ($F3/3 = 1·1$), while the difference between the effects of pitressin and pitocin gives a value of $F1/3 = 9·1$, which is probably significant.

Fig. 4 shows the maximum weight increase of the animals after injection of the two extracts. Here again over the range studied the effect is almost uniform for all doses ($F 3/3 = 3{\cdot}8$), while pitressin and pitocin are significantly different in their effects ($F 1/3 = 53{\cdot}4$).

At first sight it would appear impossible for the anti-diuretic effect of pitocin to be due to the pressor fraction which it contains. As before, the effects of pitocin

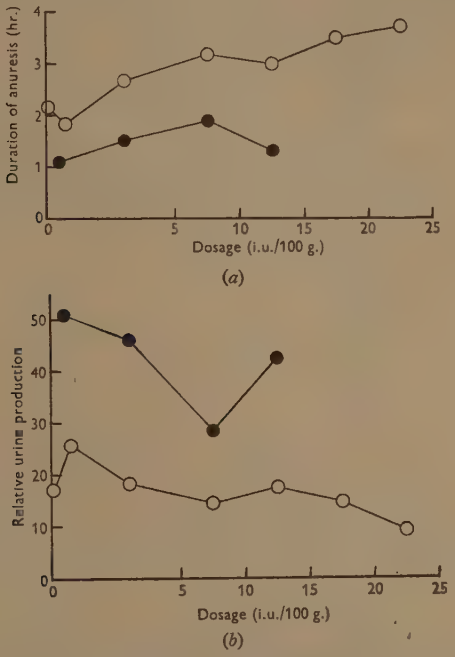


Fig. 3. (*a*) The duration of anuresis; and (*b*) the relative urine production after injection of various dosages of pitressin (open circles) and pitocin (filled circles).

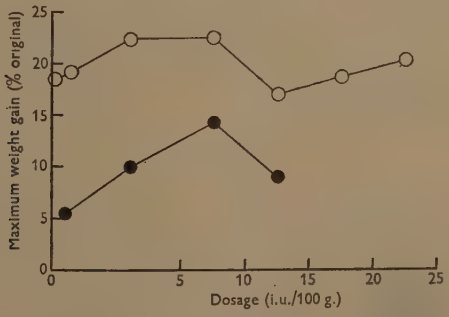


Fig. 4. Maximum increase in weight of toads after injection of various dosages of pitressin (open circles) and pitocin (filled circles).

at 0·5–1 and at 5–10 i.u./100 g. must be compared with those of pitressin at 0·1 and 0·5–1 i.u./100 g. respectively. In addition, a comparison may be made of the effects of pitocin at 10–15 and pitressin at 1–5 i.u./100 g. The figures for relative urine production are:

Pitocin: 0·5–1 i.u.; 51·0 ± 10·30
Pitressin: 0·1 i.u.; 17·2 ± 3·80

Pitocin: 5–10 i.u.; 28·4 ± 6·07
Pitressin: 0·5–1 i.u.; 25·8 ± 5·92

Pitocin: 10–15 i.u.; 42·5 ± 8·42
Pitressin: 1–5 i.u.; 18·3 ± 2·51

The difference between pitocin and pitressin is highly significant in the first case ($P < 0·003$); in the second case it is not significant, and in the third case the difference is significant ($P < 0·01$). For the duration of anuresis the figures are:

Pitocin: 0·5–1 i.u.; 1·1 ± 0·17
Pitressin: 0·1 i.u.; 2·16 ± 0·17

Pitocin: 5–10 i.u.; 1·9 ± 0·26
Pitressin: 0·5–1 i.u.; 1·83 ± 0·40

Pitocin: 10–15 i.u.; 1·3 ± 0·40
Pitressin: 1–5 i.u.; 2·66 ± 0·42

In the first case the difference is highly significant ($P < 0·001$), in the second case it is not significant, while in the last case the difference is probably significant ($P < 0·02$). The second comparison is probably the least reliable, since here the pitocin experiments include one unusually low value, and the pitressin experiments one particularly high value. Since the difference between the effects of pitocin and pitressin, both on duration of anuresis and on relative urine production, are highly significant at the lowest and significant at the highest pairs of dosages compared, it would appear to be impossible to attribute the effects of pitocin solely to its pressor fraction content. Moreover, the effect of pitocin is less, not greater, than is to be expected on the basis of its pressor fraction content alone. This suggests the possibility that pitocin may antagonize the action of the pressor fraction. If this is so, then the anti-diuretic effect of pitocin will be less, and the urine flow therefore greater, than would be expected on the basis of its 5–10% pressor fraction content. Experiments to test this point were therefore carried out.

(4) *Pitocin-pitressin antagonism*

The first point investigated was whether a mixture of pitocin and pitressin gave the same effect as an equivalent dose of pituitrin. Experiments were done at a dosage of 2·5 i.u./100 g. The results obtained in a series of seven experiments using pituitrin and seven experiments using a mixture of pitocin and pitressin are given in Table 3. There is no significant difference either in rate of water uptake for the first hour only and for the total period, or in relative urine production and

duration of anuresis. A mixture of pitocin and pitressin therefore has the same effect as an equal dose of pituitrin.

The values found using the mixture of 2·5 i.u. pitocin + 2·5 i.u. pitressin were then compared with those previously found using pitressin alone at a dosage of 1–5 i.u./100 g. The figures for pitressin alone are included in Table 3. The relative water uptake, both for the first hour and for the total period, is greater after the injection of pitocin + pitressin than it is after the injection of pitressin alone. The differences in response are significant, P being $< 0{\cdot}01$ for the first hour and $< 0{\cdot}001$ for the total period. This is in agreement with the results discussed in § 2, in which the effect of pitocin on water uptake was found to correspond with what is to be expected on the basis of its pressor fraction content.

Table 3. *Effects of pituitrin, pitressin and pitressin + pitocin*

(*Note:* 1 i.u. of pituitrin contains 1 i.u. of oxytocic + 1 i.u. of pressor activity.)

Injection	Relative water uptake		Relative urine production	Duration of anuresis	No. of experiments
	First hour	Total period			
2·5 i.u. pituitrin	253·3 ± 22·04	191·1 ± 23·77	40·4 ± 9·45	1·4 ± 0·53	7
2·5 i.u. pitressin + 2·5 i.u. pitocin	294·3 ± 20·30	230·7 ± 18·30	46·6 ± 8·42	0·7 ± 0·18	7
2·5 i.u. pitressin	197·7 ± 33·24	137·7 ± 14·36	18·3 ± 2·51	2·66 ± 0·42	6

In the case of the anti-diuretic responses, on the other hand, it can be seen that pitressin alone has a greater effect than it has when given together with pitocin. The differences are significant both for relative urine production and for duration of anuresis, the values of P being $< 0{\cdot}01$ and $< 0{\cdot}001$ respectively. Pitocin therefore significantly antagonizes the anti-diuretic effect of pitressin. Since this is so pitocin will have less anti-diuretic activity than would be expected solely on the basis of its pitressin content. This has been found to be the case. In the absence of pure extracts of the pressor and oxytocic fractions complete certainty cannot be reached; but it nevertheless seems probable that the effect of pitocin on urine formation, as well as its effect on water uptake, is due not to the activity of the oxytocic principle, but rather to the pressor fraction which it contains.

Another point of interest emerges if we compare the lengths of time for which the increased water uptake and the decreased rate of urine flow last after the injection of the two extracts. The values at the lowest dosages, which are near the threshold for an effect on water balance, are neglected in each case. The figures on which the following calculation is based are those for the individual experiments whose means are given in Tables 1 and 2. After pitressin injection anuresis lasts slightly longer than increased water uptake. The mean value for (duration of anuresis – duration of increased water uptake) is $+0{\cdot}4$ hr. The difference in duration of the two effects is, however, not significant ($P > 0{\cdot}05$). After pitocin injection, on the other hand, anuresis does not last as long as increased water uptake: the mean value for (duration of anuresis – duration of increased uptake) is $-0{\cdot}6$ hr., and the difference is significant ($P < 0{\cdot}02$). These results are in accordance with

expectation. As previously noted, the kidneys are more sensitive than the skin and react at lower concentrations. The renal effect might therefore be expected slightly to outlast the dermal effect, as may be the case after pitressin injection. With pitocin injection there is the added complication that the oxytocic fraction antagonizes the renal but not the dermal effects of the active pressor fraction. In these circumstances it is not surprising to find that the dermal effect persists longer than the renal.

IV. DISCUSSION

With the exception of Novelli (1933), previous workers have found that pitocin has a stronger water-balance effect in Anura than pitressin (Heller, 1930; Steggerda & Essex, 1934; Oldham, 1936; Boyd & Brown, 1938; Sawyer, Travis & Levinsky, 1950). The present results, and particularly the conclusion that the activity of pitocin can all be attributed to its pressor fraction content, are thus in direct conflict with the findings of almost all earlier workers. It is therefore of considerable interest to find that, while the present work was in progress, Jørgensen (1950) was independently investigating the same question. Jørgensen used a different method, different species of Anura (*Bufo bufo* and *Rana temporaria*), and his pituitary extracts were Pitupartin AB and Insipidin AB. Nevertheless, his results for *Bufo bufo* are extremely similar to those described above. In this species he finds insipidin (the pressor extract) much more active than pitupartin (the oxytocic extract); and concludes that the effect of pitupartin on water uptake can be explained as being due to its content of the pressor fraction. As regards the renal effect Jørgensen could not reach a definite conclusion, but he too finds evidence that pitupartin antagonizes the anti-diuretic action of insipidin. In *Rana temporaria* Jørgensen found the two extracts to be approximately equally active.

In view of this high measure of agreement it seems legitimate to conclude that, at least in the species *Bufo bufo* and *B. regularis*, one substance is predominantly responsible for both the dermal and renal components of the water-balance effect; and that in mammalian pituitary extracts this substance is the pressor fraction. In this connexion it is to be noted that Heller & Smith (1948) obtained from crabs' eye-stalks an extract without anti-diuretic action in rabbits and devoid of oxytocic activity which caused an increase in body weight in *Rana temporaria*. Heller & Smith conclude that the increase in weight is due to an increase in rate of water uptake, since the extract caused no very marked effect on urine flow in the frog. It should be pointed out that the activity of the frog's kidneys must have been affected, as otherwise an increased water uptake would have resulted in an increased urine output, and the animal would not have gained in weight.

If the results of those workers who have found the oxytocic more active than the pressor extract are examined it emerges that they have all used species of *Rana*. Heller (1930) used *R. esculenta*, Steggerda & Essex (1934), Oldham (1936), Boyd & Brown (1938) and Sawyer *et al.* (1950) all worked with *R. pipiens*. Novelli (1933), on the other hand, who found pitressin the more effective, was working with *Bufo arenarum*. The present work concerns *B. regularis*, and Jørgensen, who found

insipidin more active than pitupartin in *B. bufo*, found *Rana temporaria* about equally sensitive to the two extracts. These facts strongly suggest that the genera *Bufo* and *Rana* differ in their responses to pituitary extracts. *Bufo* is highly sensitive to pitressin, whereas *Rana* is much less so. Whether *Bufo regularis* reacts at all to the oxytocic fraction is not clear. No such effect can be demonstrated using pitocin, for the sensitivity to pitressin is so high that the responses to the contaminating .5–10% of the pressor fraction completely mask any possible responses to the oxytocic fraction. From Jørgensen's results the same would appear to hold for *B. bufo*.

In *Rana* the response to pressor extracts is slight, and is less than the response to oxytocic extracts. This genus might therefore be sensitive only to the oxytocic fraction, its small response to pitressin being due to the oxytocic fraction content of the extract. This possibility has been discussed and rejected by Heller (1945), and is moreover not compatible with Jørgensen's finding that *R. temporaria* responds approximately equally to the two extracts. If it were true, pitupartin should have been five to ten times as active as insipidin. Further investigation of this question is desirable.

The biological significance of this physiological difference between the two genera is probably to be found in the greater degree of terrestrial adaptation shown by toads. Jørgensen has shown that *Bufo bufo* is more sensitive to anuran pituitary extracts than is *Rana temporaria*. He concludes that the secretion of 'water-balance principle' by the posterior pituitary is of biological significance in the Anura, and that there is a correlation between the importance of such pituitary control of water balance and the habitats of the various species, a high development of such control being characteristic of those species living in the driest habitats.

In this connexion the work of Howes (1940) is of interest. Howes studied the water-balance reaction in developmental stages of *Bufo bufo bufo* and found that the response to pituitary extracts was not shown by the larvae, but developed gradually as metamorphosis took place. His experiments were carried out on various stages up till 30 hr. after tail resorption had been completed. Although Howes worked mainly with unfractionated pituitary extract he also used pitressin and pitocin in 'confirmatory experiments'. No details are given, but he remarks that 'the pitocin was more active' than the pitressin. Since Jørgensen (1950) has found that in fully grown specimens of this species the pressor extract is the more active it would appear that the high sensitivity to pitressin characteristic of the genus is developed only at a later stage than those dealt with in Howes's experiments. It is tempting to see in this an indication that the high sensitivity of *Bufo* to the pressor fraction is the most recently acquired specialization of the hormonal mechanism of water-balance regulation.

The high sensitivity of the various species of *Bufo* to pitressin is therefore probably a reflexion of the importance of pituitary regulation of water balance in this genus. The results of the present investigation on *B. regularis* are in harmony with this view. *B. regularis* is a species which can exist for long periods without access to ponds or streams. In such circumstances the ability rapidly to increase

its rate of water uptake is of advantage to the animal, since it will be able to take up water readily when rain or dew provide a temporary supply.

Since *Bufo* shows a higher sensitivity than *Rana* both to mammalian and to anuran pituitary extracts the former genus is preferable to the latter as a test animal in any experiments designed to find out whether any preparation does or does not possess the power of eliciting the water-balance effect in Anura.

V. SUMMARY

1. In *Bufo regularis* the injection of either pitressin or pitocin is followed by an increase in the rate of water absorption through the skin, together with a marked decrease in urine flow. The response to pitressin is greater than that to pitocin.

2. The effect of pitocin in increasing water uptake can be attributed to the activity of the 5–10% of the pressor fraction which it contains.

3. A mixture of pitressin and pitocin has the same effect on water balance as a corresponding dose of pituitrin. Pitressin alone has a greater anti-diuretic effect than it has when pitocin is added.

4. Taking into account the antagonistic action of pitocin to pitressin it is possible to account for the anti-diuretic action of pitocin in terms of its pressor fraction content.

5. It is concluded that the pressor fraction is the main factor responsible for both the dermal and the renal components of the water-balance effect produced in *B. regularis* by injection of mammalian posterior pituitary extracts. This conclusion is discussed in relation to the findings of other workers.

This work was carried out during the tenure of a Research Grant from the South African Council for Scientific and Industrial Research.

REFERENCES

BOYD, E. M. & BROWN, G. M. (1938). Factors affecting the uptake of water by frogs when injected with extract of the posterior hypophysis. *Amer. J. Physiol.* **122**, 191–200.

EWER, R. F. (1950). The effect of pituitrin injection on the water balance of *Bufo regularis* Reuss. *J. exp. Biol.* **27**, 40–9.

HELLER, H. (1945). The effect of neurohypophysial extracts on the water balance of lower vertebrates. *Biol. Rev.* **20**, 147–57.

HELLER, H. & SMITH, B. (1948). The water-balance principle of crustacean eye-stalk extracts. *J. exp. Biol.* **25**, 388–94.

HELLER, J. (1930). Über die Wirkung der getrennten Hypophysenhinterlappenhormonen auf die Wasseraufnahme beim Frosch. *Arch. exp. Path. Pharmak.* **157**, 323–9.

HOWES, N. H. (1940). The response of the water-regulating mechanism of developmental stages of the common toad, *Bufo bufo bufo* (L.) to treatment with extracts of the posterior lobe of the pituitary body. *J. exp. Biol.* **17**, 128–38.

JØRGENSEN, C. B. (1950). The amphibian water economy, with special regard to the effect of neurohypophyseal extracts. *Acta physiol. Scand.* **22**, Suppl. 78, 1–79.

NOVELLI, A. (1933). Extrait postéro-hypophysaire et imbibition des batraciens. *C.R. Soc. Biol., Paris*, **112**, 506–7.

OLDHAM, F. K. (1936). The action of the preparations from the posterior lobe of the pituitary gland upon the imbibition of water by frogs. *Amer. J. Physiol.* **115**, 275–80.

SAWYER, W. H., TRAVIS, D. F., & LEVINSKY, N. G. (1950). Identity and specificity of frog water-balance principle of posterior pituitary extract. *Amer. J. Physiol.* **163**, 364–9.

STEGGERDA, F. R. & ESSEX, H. E. (1934). Comparative study of effects of preparations of posterior lobe of pituitary gland on water interchange in frogs. *Proc. Soc. exp. Biol., N.Y.*, **32**, 425–8.

THE TECHNIQUE OF FREE SKIN GRAFTING IN MAMMALS

By R. E. BILLINGHAM* and P. B. MEDAWAR

From the Department of Zoology, University of Birmingham

(*Received 2 March* 1951)

(With Plates 5–7 and One Text-figure)

1. INTRODUCTION

In recent years, skin grafting has been used for the study of a wide variety of biological problems. Some of these problems relate to the properties of skin itself —e.g. pigmentation (Billingham, 1948), hair growth, sensitivity (Haxthausen, 1948), the innate 'racial' differences between the several varieties of epidermal epithelia (Billingham & Medawar, 1948*a*, *b*, 1950) and the contributions of dermis and epidermis to the formation of chemically induced tumours (Billingham, Orr & Woodhouse, 1950). With other problems, skin has been used in preference to other tissues because it it is more accessible or easier to handle, or because it gives less ambiguous answers; among these may be mentioned tissue transplantation immunity (Gibson & Medawar, 1943; Medawar, 1944, 1945, 1946*a*, *b*, 1948*a*, *b*), the measurement of the degree of homozygosity of inbred lines (McDonald & Medawar, unpublished), and the resistance of living cells to procedures such as anaerobic cultivation (Medawar, 1947) or freezing and drying (Billingham & Medawar, 1951).

The peculiar advantages of skin for grafting purposes are, briefly, these: skin is the only tissue which may easily be grafted 'orthotopically', i.e. to an anatomically natural environment; the fate of skin grafts may be watched from day to day and their behaviour easily checked by repeated biopsy and histological examination without prejudice to the well-being of the recipient; and skin may be so grafted as to self-indicate its own survival or death by the presence or absence of epithelial outgrowth from it.

The purpose of this paper is to set forth certain procedures and principles which have emerged from the authors' experience with skin grafting in rabbits, guinea-pigs and mice. The rather special problems of grafting in cows (Anderson, Billingham, Lampkin & Medawar, 1951) and monkeys (Krohn, unpublished) will be dealt with elsewhere.

The union of a skin graft with its bed is a process of healing by 'first intention', i.e. by the direct union of immediately apposed raw surfaces, and as a general rule grafting will be successful if the graft is not too thick and if it is held by light but firm pressure upon a bacteriologically clean and adequately vascular bed until primary union is complete. The greater part of this paper is concerned with

* British Empire Cancer Campaign Research Fellow.

describing in sufficient detail how these conditions can be fulfilled. Only 'free' (as opposed to pedicle or flap) grafts will be dealt with, i.e. grafts which at some stage have been wholly severed from the body; but among these it is convenient to distinguish between two sorts: (*a*) *fitted grafts* (Pl. 7, figs. 17, 19, 21–23), which exactly, or almost exactly, fill the defects into which they are transplanted, and (*b*) *open-style grafts* (Pl. 5, figs. 4–8; Pl. 6, figs. 12–14), in which more or less of the skin defect is left uncovered by grafted skin. The healing of such incompletely covered defects is achieved partly by outgrowth of skin epithelium from the grafts and ingrowth of epithelium from the edge of the raw area, and partly by the generalized contraction of the wound as a whole. Open-style grafting therefore entails a mixture of healing by first intention and by 'second' intention—i.e. (in this situation) by the progressive resurfacing of the lesion by epithelial migration. It is of particular value when the mere survival of the graft, as indicated by outgrowth from it, is the chief experimental issue.

2. THE ANATOMY OF MAMMALIAN SKIN

The integument of the mammal consists of the following layers: (*a*) the epidermis, and its appendages (hairs and glands) lying in (*b*) the dermis or corium, consisting mainly of stout collagen fibres in three-dimensional packing, and containing (under normal conditions) a sparse resident mesenchyme cell population of fibroblasts and histiocytes.

The epidermal appendages, which do not normally abut below the base of the dermis, lie within special basket-works of fine collagen and elastin fibres. Apart from these, the dermo-epidermal interface is by no means a plane surface; looked at from the inner side, the epidermis may be seen to be thrown into one or another of a variety of hill-and-valley patterns that vary from one part of the body to another. The dermal prominences that fit into the epidermal valleys—obviously the one bears the negative imprint of the other—are the so-called *dermal papillae*. Each dermal papilla contains a tuft of blood and lymphatic capillaries, and the collagen fibres are smaller and in more open packing than elsewhere. Fine elastic fibres are particularly concentrated at the superficial levels of the dermis and are thought to be responsible for holding the epidermis in place.

(*c*) *The superficial fasciae.* In the rabbit, the superficial fasciae are revealed by dissection as a number of superimposed planes of connective tissue in which the collagen fibres lie with their long axes in a plane parallel to that of the integument. Thanks to the absence of fibres running perpendicularly to the skin surface, the skin of the rabbit may easily be 'split' off at the level of these fascial planes. In the mouse and the guinea-pig, however, the superficial fasciae are represented by a layer of fatty tissue, the *panniculus adiposus*, which is rather firmly united to the dermis above and the layer of striped muscle below. The skin of these animals does not therefore split away 'naturally' below the dermis, but must be carefully dissected away.

The principal arteries, veins, lymphatics and nerves of the skin run in a direction parallel to the plane of the skin surface and lie between the superficial fasciae (or

panniculus adiposus) and (*d*) the *panniculus carnosus*, the layer of striped muscle responsible for skin-twitching movements: this layer is absent from man except in the muscles of the jaw and of facial expression. In other mammals it thins out and eventually stops about half way down the limbs.

The fibres of the panniculus carnosus are bound together by an epimysium above and below; the internal epimysium is united to the body wall by very loose areolar connective tissue which allows the integument as a whole to be freely mobile.

A skin graft consists of the epidermis and more or less of the dermis; the graft bed should as a rule be cut down to the vascular fascial planes immediately overlying the panniculus carnosus, leaving the principal vessels of the skin intact (Pl. 5, fig. 4; Pl. 6, fig. 12).

Skin shows considerable variation of minute anatomy from one part to another of the same animal; e.g. the epidermis is thicker and more deeply stratified where the coat is sparse, as it is on most mammals' ears. The dermis of ear skin is thin and the superficial fasciae are fairly loose, so that thin grafts are easier to cut from the ears than elsewhere. These regional variations, and the differences between species mentioned earlier, govern the manner and the ease with which skin grafts of a chosen type may be cut from one part or another of a particular donor. Over and above these mainly anatomical differences, there are also innate differences between the various races of epidermal epithelia—'innate' in the sense that the distinctive properties of the epithelia are conserved indefinitely after grafting to anatomically unnatural positions. Such differences may be used as self-markers to distinguish a graft from the skin that surrounds it (see § 5).

3. ANAESTHESIA

General anaesthesia is essential for grafting operations on small agile laboratory mammals, and it is desirable for the earlier changes of dressings. Nembutal supplemented by ether has been generally satisfactory; ether alone is practicable with the rabbit, but mice of certain strains and guinea-pigs are apt to have spasms or rigors unless the plane of anaesthesia is very attentively controlled.

Nembutal is commercially dispensed as a solution in 10% alcohol containing 1 gr. (=0·065 g.) per ml. The dosages we have found satisfactory are: for rabbits, 1 ml./5 lb. or 2 kg. body weight—administered through the marginal ear vein; for guinea-pigs, 1 ml./lb. body weight of Nembutal solution diluted one in five with Ringer's solution or normal saline, administered intraperitoneally; for mice, 0·1 ml./10 g. body weight of a solution diluted to one part in ten with normal saline, administered intraperitoneally.

The removal of biopsy specimens under ether alone is made easier by infiltrating the graft bed with 0·25–1·0 ml. of a local anaesthetic, such as 2% ethocaine hydrochloride. Local anaesthetics are as a rule commercially dispensed in solutions containing adrenalin, which helps to check bleeding.

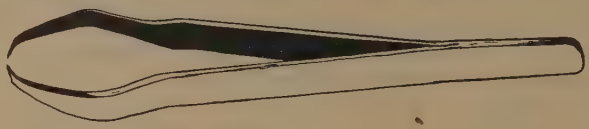
The use of Nembutal makes it desirable to operate on a heated table, to help maintain normal body temperature.

4. PREPARATION OF SKIN; INSTRUMENTS; ASEPSIS; DRESSINGS

For graft donor and recipient areas alike, hair should be cut or mechanically clipped from an area considerably larger than that which will be included in the actual operation field. The clipped area should then be thoroughly lathered with soap and shaved clean, with an open or 'Durham Duplex' razor. Ear skin may be shaved with an ordinary or miniature safety razor. The excess of soap may be removed with surgical spirit. Hair-bearing skin cannot be completely sterilized, even after shaving, but it can be adequately cleaned by swabbing with 0·1% CTAB. (cetyltrimethylammonium bromide, 'Cetavlon') in 70% alcohol. All the operations described below may now be done by 'No touch' technique, and sterile gowns or rubber gloves are therefore needless refinements.

The only donor area for which this treatment is too drastic is rabbit's general body skin, because of the thinness of its epidermis, but the damage caused to the epidermis by shaving is so soon repaired as to be of no consequence unless a special study is being made of grafts of only 4–6 days' standing.

For ordinary grafting operations, the following instruments should be available: scalpels with blades 11 (14 or 16), 12, 15 and 21; 5 and 3 in. (very fine) dog-toothed forceps; watchmaker's forceps; 'pinch forceps' (Text-fig. 1), with very sharp points,



Text-fig. 1.

which can be made by bending in the tips of watchmaker's forceps; fine curved scissors; stout straight scissors; ordinary blunt forceps. Grafts should be kept awaiting use by laying them raw side down in small Petri dishes fitted with filter papers damped with sterile Ringer's solution. Plenty of small sterile gauze swabs, most usefully squares of 5 cm. length of side, are needed during operations.

Instruments should be sterile and should be laid when not in immediate use on sterile cloth or on a glass plate sterilized with 5% Dettol. Stainless steel instruments may be sterilized without deterioration by boiling for 5 min.; plated instruments and scalpels are best sterilized in firmly corked test-tubes by dry heat (140–150° C. for 45 min.). The corks will not blow out if each is pierced by a narrow cylindrical hole firmly plugged with cotton wool. Glassware is also sterilized by dry heat.

Skin grafting requires the use of a number of special dressings. Of these, plaster-impregnated bandage is best bought commercially ('Gypsona'), but vaselined gauze (*tulle gras*) is best home-made. Tulle gras for larger animals (Pl. 5, fig. 6) is made by cutting fine open-wove bandage into rectangles of the appropriate size, packing them down firmly in a glass or metal box, drying in the oven, and then infiltrating with hot vaseline, the level of which should reach the uppermost layer of bandage. Complete impregnation and sterilization may then be achieved by baking the entire

vessel at 140–150° C. for an hour or more. The same technique, with the substitution of muslin for open-wove bandage, makes a very fine-meshed tulle which, being easy to cut into small squares and more adaptable in shape, is particularly suitable for mice.

The adhesives that are required are surgical spirit gum (e.g. Benzo-Mastiche, Mastisol), available from surgical supply houses, and a rubber latex cement such as Copydex.

5. THE PREPARATION OF GRAFTS

Two sorts of graft are useful in experimental work: (*a*) the pinch graft, so called from the manner in which it is cut, and (*b*) the split-thickness or Thiersch graft, named after a French pioneer of plastic surgery.

(*a*) The *pinch graft* is the easier to cut. The skin of the chosen donor area is raised into a cone or tent with pinch forceps or fine dog-toothed forceps and sliced off by firm horizontal strokes with a no. 12 scalpel (Pl. 5, fig. 2). Its shape is round or oval, and its diameter (anything from 3 to 12 mm.) clearly depends on the mobility of the skin and the height to which the tent or cone has been raised. Small pinch grafts are button-shaped, and taper from the full thickness of the dermis centrally to the thickness of the epidermis round their edges. Larger pinch grafts, particularly those cut from the ear, have bevelled edges but are of full dermal thickness over the greater part of their area. To expedite healing, the areas from which pinch grafts are cut should be so spaced that each is separated from its neighbour by at least 1 mm. width of normal skin.

Pinch grafts cut from rabbit's or guinea-pig's skin automatically split from their substratum at the required thickness. With mice, however, the binding of the layers of skin is such as to make it very difficult to avoid including the fatty layer and the panniculus carnosus beneath it. It is *essential* for sound healing that both these unwanted layers should be snipped off with curved scissors, or scraped away with firm strokes of a no. 15 scalpel.

(*b*) The *Thiersch graft* (Pl. 5, fig. 1; Pl. 6, fig. 10) is thinner than the pinch graft and of a uniform thickness, comprising the epidermis and the superficial layers of the dermis.* Thiersch grafts may be cut from the general body skin by holding it taut over the fingers and slicing off a thin shaving of even thickness with a no. 11 (or 14 or 16) straight-edged scalpel. Smearing the donor area with a thin film of sterile vaseline helps the cutting and later handling of such grafts. Trimming to a chosen shape is done after removal. The ear (Pl. 5, fig. 1) is a particularly favourable source of Thiersch grafts, because of the thickness of the epidermis and the sparseness of its coat of hairs. Rectangular grafts of a predetermined size and shape can be cut from the ear by defining their outlines by very light incisions with a no. 15 scalpel before the sheet is sliced off.

* Plastic surgeons now use the term *split-thickness* graft to describe one comprising the epidermis and some but not all of the dermis, and confine the term Thiersch graft to the thinnest split-thickness graft which it is practicable to cut. The present authors propose to use the term Thiersch graft in its wider generic sense, since they have used 'split skin grafts' to describe grafts of pure epidermis, i.e. epidermal sheets from which the dermis has been wholly removed.

Pinch grafts, being thicker than Thiersch grafts, take a little longer to heal soundly, but give cosmetically and functionally better results when they do: the hairs that grow from them are of normal (and invariably graft-specific) colour, density and orientation (Pl. 7, figs. 21, 22). Thiersch grafts, which do not contain the hair follicle bases, sometimes fail to regenerate a full normal crop of hairs.

It is obviously impossible to cut Thiersch grafts of adequate size from the very thin ears of mice, but it is quite easy to strip away sheets of skin having the same composition. Two straight but divergent scalpel incisions starting at the base of the ear are made down to the level of the cartilage so that, with the margin of the ear as its third side, the incisions make an equilateral triangle with its apex at the base of the ear. By grasping the skin with fine forceps at the apex the entire triangle may then be peeled away and later trimmed to whatever shape is wanted.

The donor areas of pinch grafts or Thiersch grafts may be dusted thickly with sterile sulphadiazine powder from an insufflator. The powder has a usefully haemostatic function. Other dressings are unnecessary and probably undesirable. The donor areas of Thiersch grafts heal with almost spectacular speed by the upward and outward migration of epithelium from the bases of truncated hair follicles; those of pinch grafts form dry protective scabs, and wound closure is brought about partly by epithelial ingrowth but mainly by general contraction. Pinch graft donor areas remain recognizable by small scars.

It is not obligatory to use skin grafts within a few minutes of their being cut: they are known to remain viable for weeks in the refrigerator and probably for ever at the temperature of liquid air: but if the early post-operative history of a skin graft is of consequence it is probably as well, for uniformity's sake, to use all grafts within a few hours of their removal.

The choice of donor area: 'labelled' grafts. It is sometimes of vital importance that grafted skin should remain distinguishable without possibility of error from the skin of the recipient area around it.

With body skin grafts, a difference of hair colour is the most conspicuous label; differences of hair length or density (as, for example, between belly and back) may also be used. In the guinea-pig, where the superficial epidermis may be pigmented, differences of skin pigmentation as such may also be made use of (Pl. 7, fig. 19). There is only one drawback to the use of colour markers: the loss of pigmentation by destruction of melanophores does not always entail the death of epidermal cells, and, conversely, epidermal cells may sometimes be caused to die under conditions in which the pigmentation associated with them persists (Billingham & Medawar, 1950).

Other innate differences may be used as alternatives to marking grafts by their colour. In mice, for example, Thiersch grafts cut from the skin of the tail remain easily recognizable as such when grafted to the skin of the chest (Pl. 6, figs. 13–16). Ear skin also preserves its specificity of type (Pl. 7, fig. 19), and in guinea-pigs the hairless and rapidly proliferating epidermis of the sole of the foot (Pl. 7, fig. 17) provides a useful and conspicuous marker.

Pure epidermal grafts. Crude pancreatic extracts and commercial trypsin powders

contain an enzyme (probably an elastase: cf. Balo & Banga, 1949) that causes the epidermis to free itself without damage from the dermis (Medawar, 1941). Pure epidermal or *split skin grafts* (Pl. 7, fig. 20), as we call them, are therefore easy to prepare from ear skin Thiersch grafts, in which the epidermis is thick and there are insufficient hair follicles to interfere with the clean fission of dermis from epidermis. Some crude trypsin samples contain further enzymes that cause maceration of the epidermal layer, and these make possible the use of epidermal cell suspensions as grafts. Alternatively, the intact epidermal sheet may be floated upon a mixture of 1 vol. of 4% sodium citrate with 4 vol. of normal saline.

Suitable 'skin splitting' solutions may be prepared by dissolving 0·25–0·5 g. commercial trypsin powder in Ringer-bicarbonate or Ringer-phosphate (pH 7·4–7·8) containing 1:100,000 phenol red, and filtering the opalescent suspension so formed first through two thicknesses of ordinary filter-paper and then through a sterile Seitz filter. (If Ringer-bicarbonate is used, filtration under negative pressure must of necessity be slow.) The filtrate may be stored in sealed 10 ml. glass ampoules in the refrigerator; it retains its activity for at least 3 months.

Skin that is to be split should for preference be thin, and it is essential that it should be of even thickness. A convenient procedure is to cut Thiersch shavings from the thinly vaselined skin of the dorsum of the ear and to gum them, outermost side downwards, on to a vaselined cover-slip. The whole cover-slip is then incubated in a 37° C. water-bath under sufficient trypsin solution to cover it. When splitting is complete, the dermis can be simply peeled off by grasping it at one corner with watchmaker's forceps; tests should be made after 15 min. incubation and at 10 min. intervals thereafter to make certain that incubation is not unnecessarily prolonged.

For ordinary grafting purposes, pure epidermal grafts are of very limited usefulness, because, if grafted to freshly prepared or even to already granulating raw areas (see below), they do not prevent the closure of the graft bed by generalized contraction.

6. PREPARATION OF RECIPIENT AREAS AND TRANSPLANTATION

The best recipient area for skin grafts is the skin of the chest dorsally or laterally, where the ribs or backbone provide a firm substratum. The lateral thoracic and mammary arteries and their branches, running in the superficial fasciae, provide a perfectly adequate overlapping blood supply throughout the entire region. Venous and lymphatic drainage is in a forward direction, towards the axilla.

Fitted grafts. Fitted pinch grafts (Pl. 7, figs. 17, 19, 21–23) are the easiest to do; the operation amounts to no more than placing one pinch graft in the skin defect or crater left by cutting another. If a pinch graft is replaced in the hole from which it was cut, it no longer fits closely, for the contraction of the graft and the slight gaping of the wound cause it to be surrounded by an annulus of unsurfaced tissue. A good fit, therefore, entails the cutting from the recipient area of a pinch graft rather smaller than the one intended to take its place.

A Thiersch graft is more usually square or rectangular than oval or round; if it is to be an exact fit, therefore, the edges of its intended bed must be defined by

incisions of suitable depth with a no. 15 scalpel, after which the skin rectangle lying within the incisions may be removed as a pinch graft is cut or by slicing it off with a straight-edged scalpel. For most purposes, however, the exact fitting of a rectangular Thiersch graft is a needless refinement. It is good enough to plant it into an oval or circular bed, where wound contraction and epithelial outgrowth will cause an approximation of the edges that is for all ordinary purposes cosmetically adequate.

Graft beds of the type that are left behind by what is in effect the procedure of cutting pinch grafts are very easy to prepare in rabbits and not much less easy in guinea-pigs. In the mouse they are fairly difficult to prepare, because of the close binding of fatty and muscular layers, and it is essential, for good healing, that the panniculus carnosus of the graft bed should be left intact. The best method of several that have been given lengthy trial is to remove a very small pinch graft from one corner of the future raw area, after which the skin edge may be picked up with pinch forceps and dissected in parallel strips from the vascular planes overlying the panniculus carnosus (Pl. 6, figs. 11, 12).

Open fit grafts. When a graft is deliberately transplanted to a bed that is too large for it, so that the rim of the graft is separated by a stretch of raw skin bed from the margin of the raw area, it is said to be in 'open fit' (Pl. 5, fig. 5; Pl. 6, fig. 13). In most animals the only workable method of grafting in open fit is to graft a relatively small pinch or Thiersch graft into the raw area left by removing a relatively large pinch graft; but in the rabbit (and, less easily, in the mouse) open areas may be made large enough to accommodate quite a number of separate grafts. In a rabbit weighing upwards of 2 kg. it is possible to cut a rectangular graft bed as much as 7×6 cm. in area (Pl. 5, figs. 3–5). The outline of such an area is first defined by shallow, clean incisions with a no. 15 scalpel; one of the four corners is then grasped with dog-toothed forceps and gently pulled towards the diagonally opposite corner. The skin within the boundary incisions can then be dissected away at the level of the superficial fascia by a series of smooth, almost horizontal, incisions with a no. 21 scalpel applied to the reflected flap of skin at the level at which the dermis is united to the superficial fascia. All residual tags of skin, such as might house the base of a hair follicle, must be painstakingly dissected away. The area has been cut too shallow or too deep if the pattern of the superficial veins and arteries (which run concomitantly) is not visible in fine detail. The panniculus carnosus should nowhere be perforated.

Grafts may be transplanted to the surface of such a raw area immediately after it has been made (Pl. 5, fig. 5); alternatively, the area may be prepared (and suitably dressed: see below) 3–5 days before grafts are transplanted to it. The formation of granulation tissue expedites primary healing and vascularization. This is advantageous when the grafts are obliged to be thick or when they have been treated *in vitro* in such a way as may prejudice their chances of survival. Healthy granulation tissue is dryish, has a fresh, pink colour, and takes on the imprint of its immediate dressing, tulle gras (see below).

In the mouse, raw areas up to 1 cm.^{2} in size may be prepared by extending a bed

of the type cut to receive fitted grafts (Pl. 6, figs. 11, 12). No attempt should be made to prepare large granulating areas in guinea-pigs unless their intake of vitamin C is of known adequacy.

The act of grafting itself amounts to no more than putting the grafts wherever they were intended to go (Pl. 5, fig. 5; Pl. 6, fig. 13). Some pains must be taken to see that Thiersch grafts lie flat. Since wet grafts are apt to be slippery, it is helpful to blot them dry on gauze before grafting. It is not harmful, and may be helpful, to lay the grafts on a bed containing a small quantity of freshly exuded blood, but they should not be laid on clotted blood. The dusting of an open-style bed with sterile sulphadiazine powder from an insufflator (a pepper pot is not so good: the powder is slightly hygroscopic) checks capillary bleeding, and the powder probably has an antiseptic action.

Pure epidermal grafts are delicate and are best handled by flotation. The vaseline that will have been used to grease their surfaces causes them to float cuticle side up and perfectly flat. A drop of Ringer's solution is placed on their intended bed, and to this they are transferred from the Petri dish in which they await grafting by lifting them up with a thin glass rod with an L-shaped tip. The excess of fluid on the graft bed should be drained away with sterile gauzes or filter paper.

Pure epidermal grafts (Pl. 7, fig. 18) will unite, or at least appose themselves firmly and in a functionally adequate way, either to a freshly cut raw skin bed or (better) to a granulating raw area; but for reasons that will be made clear later it may be best to graft them to a 'half-thickness' bed, such as is left behind when a Thiersch graft is removed. Such beds retain the greater part of the dermal collagen, and do not contract.

Dressings. Tulle gras is the best immediate dressing for skin grafts of all types on all animals. The sheet should be cut to such a size that it overlaps the graft bed all round (Pl. 5, fig. 6; Pl. 6, fig. 14).

The purpose of the outer dressings is to protect the graft physically, to hold it down with a slight perpendicular pressure, and to prevent side-slip. The means by which these conditions can be fulfilled vary from one animal to another.

Rabbits. With open style grafts, particularly in thick-skinned animals, a pressure pad consisting of several thicknesses of surgical gauze cut to the size of the skin defect should be laid over the tulle. The entire thorax should then be firmly wound with 2 yards of 3 in. bandage, great care being taken to keep the pressure on the grafts perpendicular to their surfaces, so as to prevent side-slip. To make the bandage stiff and self-adherent the chest is finally wound with an 18–24 in. length of plaster-impregnated bandage (e.g. 'Gypsona') (Pl. 6, fig. 9). The plaster bandage should be given 5 min. to set before the animal is handled; thereafter it needs no special treatment. With fitted grafts the pressure pad may be omitted.

Guinea-pig. The guinea-pig is more nearly cylindrical in shape than the rabbit, and dressings of the type just described may well slip and cause the grafts to slip with them. This shortcoming may be abolished by painting the skin round the operation field with a surgical spirit gum (Mastisol) after the tulle gras has been applied. Bandage and plaster may then be wound round the thorax with the pre-

cautions just described; the gum sticks the bandage firmly to the skin. An alternative to the use of Mastisol is to cover the tulle with an overlapping square or rectangle of adhesive plaster; this achieves the same effect, but is undesirable because it may cause a severe inflammation of the skin.

For a young adult guinea-pig (approx. 500 g.) 18 in. lengths of both 1½ in. plain and 1½ in. plaster bandage should be sufficient.

Mice. In mice the use of plain bandage may be dispensed with; plaster bandage is wound directly round the chest, where it sticks firmly to the hairs (Pl. 6, fig. 15). A 7 in. length of ½ in. wide plaster is sufficient for a 20 g. mouse. This method is trouble-free and very reliable. After drying, the plaster sheath can be painted with picric acid to discourage attempts to gnaw it away. In mice, the use of a small pressure pad between plaster and tulle is desirable only with open-style grafting to relatively large raw areas.

7. INSPECTIONS AND POST-OPERATIVE DRESSINGS

The first inspection and change of dressings should not, if possible, be done before the fourth day after operation, and may well be deferred until the tenth; it is a safe rule that change of dressings should be done no more often than the plan of an experiment requires. Post-operative dressings can be relatively light; a pressure pad may be dispensed with, except perhaps as an absorbent of serous exudate with open style grafts; tulle gras is required only so long as any raw (i.e. not yet epithelialized) tissue remains in the operation field; and as an alternative to the use of plaster the free end of the plain bandage may easily be secured by painting with latex solution.

Although new vessels have begun to penetrate free skin grafts by the 24th hour, so that there is a luxuriant growth of new blood vessels and lymphatics by the 5th or 6th day, the strength of the primary union has by no means reached its maximum at the end of the first week. For this reason, the tulle should be removed circumspectly at the first change of dressings: dried serous exudate can cause the hair stumps on the grafts to stick so firmly to the tulle that the removal of the tulle may sometimes cause a graft to come away with it. So long as any area remains uncovered by skin epithelium, asepsis should be maintained by no-touch technique and the use of sterile apparatus. Fitted grafts have become confluent with the surrounding skin well before the 6th day, and, in theory, no dressings should be required thereafter. But as the reagents used to clean the skin, and the dressings themselves, cause some irritation and itching, it is just as well to keep the dressings on for a week or 10 days longer to prevent the operation field's being excoriated by scratching.

8. THE APPEARANCES OF GRAFTS: AUTOGRAFTS

Competently transplanted skin grafts (other than pure epidermal grafts and the very thinnest Thiersch grafts) eventually reassume the appearance and properties of the skin they were cut from (Pl. 6, fig. 16; Pl. 7, figs. 17, 19, 21–22). To outward appearance, this functional and cosmetic completion of the healing process is to be expected by 20–25 days; the grafts, if of hair-bearing skin, will have grown a pelt of

hairs of graft-specific number, colour, and orientation (Pl. 7, figs. 21–22); sebaceous glands, after a period of excessive secretory activity, will be normally functional; whatever oedematous swelling may have occurred in the course of primary union will have subsided, and the graft becomes normally mobile and supple. Only microscopy will reveal certain abnormalities, such as the undue richness of the dermis and graft bed in blood vessels, lymphatics and mesenchymal cells, and other evidence of very low grade 'traumatic inflammation' which lasts until, perhaps, the 50th or 60th day after grafting.

The outward appearances of skin grafts between operation and the completion of healing now deserve some mention. A fitted graft and the 'graft centre' of the open-style graft behave alike, except, of course, that the fitted graft establishes an incisive suture line with the skin surrounding it within a few days of its transplantation.

A graft inspected at the 6th day post-operatively is soft to the touch and slightly swollen and (if of weakly pigmented or colourless skin) can be seen to have acquired a delicate pink flush—the consequence of a hypervascularity that slowly subsides from about the 8th day onwards. Though there is not likely to be much sign of it from the outside, the epithelium of a 6-day graft is violently hyperplastic; the epidermis thickens threefold or fourfold or even more, the hair follicles become keratinized trumpet-shaped cysts, and a thick layer of keratinized cells forming a sort of dead cast of the graft epithelium can eventually be stripped away, the original hairs of the graft being trapped in a life-like way within it. We call this cast the 'ghost graft'. The ghost will generally come away as a single sheet if the graft has been left undisturbed for 10 days, either by lifting it from the graft (edge first) with watchmaker's forceps, or simply stuck to the tulle.

The surface revealed by removal of the ghost graft is, in rabbits and mice, firm, white, appreciably waxy or aquafuge (hypertrophic sebaceous glands being now in action), and dotted over with the little depressions that mark the mouths of hair follicles. Guinea-pig skin, particularly ear or sole-of-foot skin, if black or dark brown to begin with, is not likely to be at any stage quite white; but pigmentation becomes transiently much paler, and even a black graft may be no more than a dusky leaden blue at its palest.

The proliferative activities of the epidermis, slowly subsiding over the following 2 weeks, leave behind an anatomical record in the thickened stratum corneum, the outer layers of which can be peeled or stripped off from time to time at inspections.

The rudiments of new hairs can be seen microscopically by the 10th or 12th day; if pigmented, they cause a shadow-like discoloration to be visible from the outside by the 14th or 16th day. The young hairs may be expected to pierce the graft surface by the 16th to 20th day. Meanwhile the epidermis settles down to a more normal thickness.

Open-style grafts increase their epithelial area by symmetrical outgrowth over what starts as, or soon becomes, granulation tissue. A 2–3 mm. wide ring of shiny white, thickly keratinized outgrowth should be visible to the naked eye by the 8th day (Pl. 5, fig. 7), and outgrowth proceeds until neighbouring grafts have

coalesced with each other and with the incoming tide of epithelium from the edges of the raw area (Pl. 5, fig. 8). Granulation tissue covered over by epithelium soon becomes collagenized, so turning into young fibrous tissue; as the collagen fibres mature and thicken, fibroblasts become fewer and individually more attenuated.

The epithelium that overlies this substratum of young fibrous tissue is chronically hyperplastic; hairs and sebaceous glands develop sparsely and very late, if at all; its attachment, lacking elastic fibres, is weak, and, unlike the epithelium of the graft centre, it may easily be peeled from its substratum. Except for odd bluntly digitate ingrowths, it keeps throughout a plane surface of contact with the fibrous tissue underlying it, and dermal papillae never form.

'Spread epithelium' of this sort is not a stable tissue. In some manner most of the spread epithelium disappears during wound contracture, and what happens to it is at present a mystery. At all events, the graft centres, which themselves expand during the contracture of the wound, eventually become bunched up together. A pure epidermal or 'split skin' graft (Pl. 7, fig. 18) is obviously comparable to the pure epidermis that spreads from an open style graft, and its ultimate fate during the process of contracture after grafting to a full-thickness skin defect is equally obscure. It manifestly survives if sufficient dermal collagen remains in the graft bed to prevent this contracture. Such a bed is that left behind by removing skin only to the thickness of a Thiersch graft. Unfortunately, its preparation entails leaving the bases of the hair follicles of the recipient area behind. The epithelium of the grafted area will thus be of dual origin.

As an animal grows, its grafts grow with it—casual observation suggests that they grow at the same rate as the skin around them, and to a final size that may exceed the size they would have reached in their original positions.

9. THE APPEARANCES OF GRAFTS: HOMOGRAFTS

Skin is not known to survive orthotopic transplantation from one individual to another of the same species unless the two individuals are (*a*) identical twins or members of a very highly inbred line; or (*b*) are dizygotic twins that have desensitized each other, presumably by the interchange of foetal cells made possible by a synchorial placenta—a process at present known to occur only in the cow (Anderson, Billingham, Lampkin & Medawar, 1951); or (*c*) unless the recipient has been treated with drugs capable of holding the homograft reaction at bay (e.g. cortisone: Billingham, Krohn & Medawar, 1951).

The intensity and quality of the reaction against orthotopic skin homografts, as judged by the epithelial survival time and the nature of the events that precede breakdown, varies somewhat from one species to another. Within any one species, it varies with (i) the antigenic relationship between donor and recipient, (ii) the quantity of skin that is grafted, (iii) the recipient's experience of grafting from the same donor, or from a donor antigenically akin to it; and (iv), presumably, with the level of circulating cortisone-like adrenal cortical steroids in the recipient. Variables (iii) and (iv) are clearly of specialized interest and import, and of the first two, the first enormously outweighs the second. In mice, with all other variables made

effectively constant, differences of antigenic relationship alone can be responsible for differences of breakdown time as great as between 5 and 80 days.

In general, the homograft reaction may be 'acute' (even though dilatory in actual onset) or 'chronic'. The chronic reaction may be such that it falls short of bringing about a complete breakdown of the homograft epithelium. Instead, it causes the epithelium to thin out and weaken in its attachment and suppresses the differentiation of glands, arrector muscles and hairs, to the accompaniment of a prolonged low-grade inflammatory reaction in the graft dermis. Very often this condition is temporary, and the graft, having lost its pelt of hairs at an earlier stage of the reaction, grows a new one and becomes generally more normal in appearance. A chronic, incomplete, reaction of this sort has been found by McDonald & Medawar (unpublished) in grafts between mice of an inbred strain which had gone some way towards achieving antigenic uniformity; by Anderson, Billingham, Lampkin & Medawar (1951) in some of their grafts between dizygotic twins in cattle; and by Billingham, Krohn & Medawar (1951) in rabbits under treatment with cortisone. The evidence from mice shows that skin grafts can override certain minor antigenic differences between donors and recipients, a fact already known to students of tumour transplantation (see below).

The epithelium of skin homografts undergoing a mild and long drawn-out breakdown reaction may be surreptitiously replaced by the surrounding epithelium of the host, while the collagen fibres of the homograft dermis persist. This phenomenon undoubtedly accounts for many mistaken claims for the success of skin homografting in human beings. To be quite sure that it has not happened, it is essential to use homografts 'labelled' by some distinctive property of hair colour or epithelial conformation.

The 'acute' reaction, which may be detected as early as the 5th or as late as the 40th day after grafting, is one which, having started, goes rapidly and progressively to completion. A homograft is perfectly capable of growing hairs and differentiating as normal skin, provided only that it lives long enough to do so.

Acute breakdown is always preceded and accompanied by an inflammatory reaction of great violence. The first sign of breakdown is the oedematous swelling of the graft (more prominent in rabbits and cows than in other species). The colour of the graft changes from light to dark pink, through brick red to various shades of yellow and brown. Its superficial epidermis now weakens, and may easily be scraped or picked away to reveal the damp, exuding surface of the graft dermis. At this stage, the migratory epithelium from an open style homograft will already have disappeared, or be represented only by some pussy cuticular debris. With fitted grafts, the perimeter becomes disengaged from the surrounding native epithelium, so that the graft acquires a free rim. When epithelial breakdown is complete, there is nothing to prevent the graft dermis drying in air and becoming reduced to a withered and blackened scab, and this it accordingly does (Pl. 7, fig. 23).

Grafts that submit to acute breakdown are almost invariably undermined by the ingrowth of native epithelium: they are very rarely overgrown. The last remnant of such a homograft is a mushroom-like object attached by a central fibrous stalk. When

this stalk is finally cut through by the advancing native epithelium the scab falls away.

While the breakdown reaction is in progress the proportion of epithelium still surviving cannot be reliably estimated by the grafts' outward appearance. Histological examination of a graft biopsy is essential. Since epithelial breakdown is progressive and takes place simultaneously and to the same degree in all the grafts transplanted on one occasion from one donor to one recipient, it follows that repeated graft sampling provides a faithful and consistent picture of the course of the breakdown reaction in the graft population as a whole.

10. A NOTE ON THE PATTERN OF GRAFTING OPERATIONS

Mention has already been made of the fact that the homografting of skin may be used to estimate the degree of genetic diversity among the animals of a chosen group. For such a purpose skin grafts are clearly preferable to, for example, subcutaneous grafts of spleen (as in the pioneer work of Little & Johnson (1922) and Bittner (1936))—partly because even autografts of spleen grow indifferently, but chiefly because the 'scoring' of the survival of spleen homografts can only be done on an all-or-none basis. Skin is also in some important ways preferable to tumour tissue, although tumours are very much the easier to work with. Skin homografts are much more sensitive to the minor antigenic differences between donor and host which tumour grafts can sometimes override (Gorer, 1942; Snell, Cloudman, Failor & Douglass, 1946), and the use of tumours for the assessment of genetic uniformity is mainly confined to testing the homogeneity of an inbred strain in some member of which a spontaneous tumour has arisen, or the degree of divergence of sublines originally derived from it. (It is an added complication that such tumours may lose some degree of antigenic specificity during the course of their maintenance by transplantation: cf. Little & Gorer (1943); Little (1947).) Moreover, all the fragments of a single tumour grafted to, say, a population of mice, are of the same genetic composition: tests for homogeneity making use of tumour grafts are therefore all of the type classified as Type D below—the antigenic composition of the graft becomes a parameter of the experiment instead of one of its most useful variables. Skin, by contrast, can be removed from any individual at will, and grafted at will to any other.

We append brief notes on the five principal patterns of homografting operation which we have employed.

Type A: complete cross-grafting. Each individual among a group of n members receives one or more grafts from each of the others, making $n(n-1)$ distinct pairings of donors with recipients. If of heterogeneous origin, the grafts borne upon any one recipient will elicit reactions of different intensities and survive for different lengths of time. If this property is to be made use of, all the grafts must be transplanted to a chosen recipient on a single occasion (i.e. in one operating session). Otherwise the grafts earlier transplanted will curtail the life of their successors to a degree which depends upon the extent of the overlap of their antigenic consti-

tutions. (Grafts of different genetic origin do in any case act to some degree synergistically when transplanted to a single recipient, for they are certain to share some antigens in common; but they can still be of sufficient disparity to survive for very different lengths of time.)

A complete cross-grafting is specially valuable when the group is presumed to be heterogeneous, and the purpose of the test is to pick out isolated instances of compatibility. The theory of the test has been discussed by Medawar (1945). Tests of types B, C, D and E are merely so many restricted variants of cross-grafting, chosen only because they yield up their information in a more economical way.

Type B: the 'ring' test. A group of animals is so operated upon that the first gives a graft to the second, the second to the third, and so on, until the ring is completed by transplanting a graft from the last to the first. This test is of special value when the group is presumed to be homogeneous, and the purpose of the test is to pick out isolated instances of incompatibility. Although any chosen ring arrangement of n animals is only one among $(n-1)!$ possibilities, any one such test *must* (with one exception of no great practical importance) single out an animal whose antigenic constitution is appreciably different from the others'. In its simplest form, the exceptional case is this. Let antigens A and B be supposed too weak to provoke an appreciable immunity when acting singly but strong enough to do so when acting simultaneously. Of three successive animals in a ring, the first may have the constitution AB, the second A alone (or B alone), and the third may lack both antigens. It follows *ex hypothesi* that no incompatibility will be revealed by the ring test in its stated form; but it would have been revealed if the ring had been so arranged that the first animal gave a graft to the third.

Type C: reciprocal interchange of grafts between animals grouped in pairs. Such a test may be used (except with cows: see below) to distinguish monozygotic from dizygotic twins or to decide whether a breeding pair are sufficiently alike to be chosen as the parents of the succeeding generation of an inbred strain. For this second purpose the test is far from exhaustive, for if a group of potential parents contains p individuals of one sex and $q\,(>p)$ of the other, only p pairings of the pq that are possible can be set up and tested by graft interchange.

Type D: parallel recipients. Grafts are transplanted from a chosen donor to two (or more) recipients. A test making use of transplanted tumours is essentially of this sort. The homograft that survives longest is borne by the individual having most antigens in common with the antigens of the donor.

Type E: parallel donors. Grafts are transplanted from two (or more) donors to a single recipient. In general, the homografts will survive for different lengths of time, and the homograft that survives longest comes from the donor that has the fewest (or the weakest) of the antigens that are not also possessed by the recipient. Anderson *et al.* (1951) point out that this is the only transplantation method that could make it possible to distinguish between monozygotic and dizygotic twins in animals such as the cow, to which tests of Type C are inapplicable. The sensitivity of the test can obviously be increased by immunizing the recipient to skin from *one* of the donors beforehand.

SUMMARY

Methods are described for the execution of free skin grafts in rabbits, guinea-pigs and mice.

Much of the work in which use has been made of the techniques described above has been generously supported over a period of years by the Department of Plastic Surgery, University of Oxford (Prof. T. Pomfret Kilner, F.R.C.S.).

REFERENCES

ANDERSON, D., BILLINGHAM, R. E., LAMPKIN, G. H. & MEDAWAR, P. B. (1951). The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity* (in the Press).

BALO, J. & BANGA, I. (1949). Elastase and elastase inhibitor. *Nature, Lond.*, **164**, 491.

BILLINGHAM, R. E. (1948). Dendritic cells. *J. Anat., Lond.*, **82**, 93–109.

BILLINGHAM, R. E., KROHN, P. & MEDAWAR, P. B. (1951). Effect of cortisone on the survival of skin homografts in rabbits. *Brit. Med. J.* 1, 1157–63.

BILLINGHAM, R. E. & MEDAWAR, P. B. (1948*a*). Pigment spread and cell heredity in guinea-pig's skin. *Heredity*, **2**, 29–47.

BILLINGHAM, R. E. & MEDAWAR, P. B. (1948*b*). 'Infective' transformations of cells. *Brit. J. Cancer*, **2**, 126–31.

BILLINGHAM, R. E. & MEDAWAR, P. B. (1950). Pigment spread in mammalian skin: serial propagation and immunity reactions. *Heredity*, **4**, 141–64.

BILLINGHAM, R. E. & MEDAWAR, P. B. (1951). The freezing and drying of mammalian skin. *Quart. J. Micr. Sci.* (in the Press).

BILLINGHAM, R. E., ORR, J. W. & WOODHOUSE, D. L. (1950). Epidermal transplantation during chemical carcinogenesis. *Nature, Lond.*, **166**, 1080.

BITTNER, J. J. (1936). The transplantation of splenic tissue in mice. *Publ. Hlth Rep., Wash.*, **51** (i), 244–7.

GIBSON, T. & MEDAWAR, P. B. (1943). The fate of skin homografts in man. *J. Anat., Lond.*, **77**, 299–310.

GORER, P. A. (1942). The role of antibodies in immunity to transplanted leukaemia in mice. *J. Path. Bact.* **54**, 51–65.

HAXTHAUSEN, H. (1948). Vitality of epidermal cells after allergic exposure. *Acta Allergologica*, **1**, 311–31.

LITTLE, C. C. (1947). The genetics of cancer in mice. *Biol. Rev.* **22**, 315–43.

LITTLE, C. C. & GORER, P. A. (1943). The genetics of cancer in mice. In *The Genetics of the Mouse*, by H. Gruneberg, pp. 311–32. Cambridge University Press.

LITTLE, C. C. & JOHNSON, B. W. (1922). The inheritance of susceptibility to implants of splenic tissue in mice. *Proc. Soc. Exp. Biol., N.Y.*, **19**, 163–7.

MEDAWAR, P. B. (1941). Sheets of pure epidermal epithelium from human skin. *Nature, Lond.*, **148**, 783.

MEDAWAR, P. B. (1944). The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat., Lond.*, **78**, 176–99.

MEDAWAR, P. B. (1945). A second study of the behaviour and fate of skin homografts in rabbits. *J. Anat., Lond.*, **79**, 157–76.

MEDAWAR, P. B. (1946*a*). Immunity to homologous grafted skin. 1. The suppression of cell division in skin grafts transplanted to immunized animals. *Brit. J. Exp. Path.* **27**, 9–14.

MEDAWAR, P. B. (1946*b*). Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. Exp. Path.* **27**, 15–24.

MEDAWAR, P. B. (1947). The behaviour of mammalian skin epithelium under strictly anaerobic conditions. *Quart. J. Micr. Sci.* **88**, 27–37.

MEDAWAR, P. B. (1948*a*). Tests by tissue culture methods on the nature of immunity to transplanted skin. *Quart. J. Micr. Sci.* **89**, 239–52.

MEDAWAR, P. B. Immunity to homologous grafted skin. III. The fate of skin grafts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Brit. J. Exp. Path.* **29**, 58–69.

SNELL, G. D., CLOUDMAN, A. M., FAILOR, E. & DOUGLASS, P. (1946). Inhibition and stimulation of tumor homoiotransplants by prior injections of lyophilized tumor tissue. *J. Nat. Cancer Inst.* **6**, 303–16.

EXPLANATION OF PLATES

PLATE 5

Fig. 1. Illustrating the technique of cutting a Thiersch graft from the dorsum of a rabbit's ear. The donor area has been shaved and treated with sterile vaseline to facilitate the cutting.

Fig. 2. Illustrating the technique of cutting a 'pinch' graft from the shaved and sterilized skin on a rabbit's flank. A small tent of skin is raised by pinch forceps and its base is cut free by means of almost horizontal incisions with a no. 12 scalpel.

Fig. 3. Showing the initial stages in the preparation of an extensive rectangular raw area on a rabbit's thorax. The outlines of the rectangle have been defined by means of vertical incisions with a no. 15 scalpel and a flap of skin is being reflected with dog-toothed forceps towards the diagonally opposite corner. By almost horizontal incisions with a no. 21 scalpel the skin is caused to split easily at the level where the dermis is united to the vascular fascial planes inmediately overlying the panniculus carnosus muscle.

Fig. 4. Showing the large rectangular recipient area ready for transplantation of the grafts. All traces of dermis have been completely removed. Note the main thoracic vessels and their branches.

Fig. 5. Showing six medium-sized pinch grafts cut from the skin of the rabbit's flank and transplanted to an extensive raw area on its chest.

Fig. 6. To show the sheet of tulle gras in position immediately overlying the grafts. The dressing is self-adherent and semi-transparent.

Figs. 7, 8. The appearance of the operation field illustrated by fig. 6 after 8 days (fig. 7) and after 10 days (fig. 8). The vessel tracks have been obscured by the formation of granulation tissue over the graft bed. Note that there are indications of epithelial outgrowth from the grafts and from the margins of the operation field at 8 days, and that by 10 days the grafts have become surrounded by annuli of well keratinized epithelium and that some have coalesced. Note the faint imprint of the tulle gras.

PLATE 6

Fig. 9. Showing the dressings in position around the rabbit's thorax.

Fig. 10. Illustrating the method of cutting a Thiersch graft from the tail skin of a mouse. The skin has been shaved and lightly smeared with vaseline to facilitate the cutting and subsequent handling of the graft.

Fig. 11. Showing the initial incision in the preparation of an extensive rectangular raw area on the thorax of a mouse.

Fig. 12. Showing the rectangular recipient area ready for the transplantation of the grafts. The lateral thoracic vessel group and its branches can be seen clearly.

Fig. 13. Showing four pigmented tail-skin autografts immediately after transplantation.

Figs. 14, 15. Showing the sheet of fine home-made tulle gras in position over the grafts (fig. 14); and the final outer dressing of plaster of Paris-impregnated bandage in position round the entire thorax (fig. 15).

Fig. 16. Showing a group of pigmented tail skin Thiersch grafts, 64 days after transplantation to the white skin of a mouse's chest. Note that the grafts have completely conserved their characteristic tail-skin appearance (cf. Fig. 10).

PLATE 7

Fig. 17. Showing a pigmented sole-of-foot graft 200 days after transplantation to a guinea-pig's chest. Throughout this period the graft has maintained all the characteristic features of sole-of-foot skin.

Fig. 18. Showing an extensive rectangular recipient area cut in the thoracic skin of the rabbit to which a series of 'pure' epidermal grafts had been transplanted 11 days previously. These grafts have clearly survived, though their individual outlines can no longer be distinguished because of the confluence of their epithelial outgrowth. Note also the ingrowing epithelium from the margins of the operation field; in some places, it has already become confluent with the epithelium from the grafts.

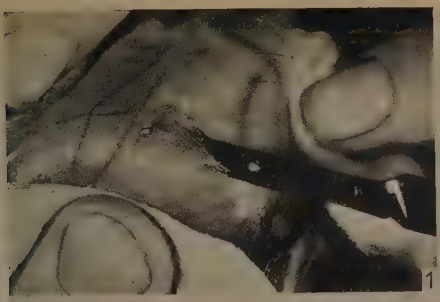
Fig. 19. Showing a black ear skin graft 1000 days after transplantation to the red skin on a guinea-pig's chest. The graft has clearly maintained its specificity (i.e. it still remains characteristically ear skin) and its margin is absolutely incisive.

Fig. 20. Showing a transverse section through a sheet of pure tail skin epidermis prepared from a Thiersch shaving from a mouse's tail by means of a tryptic digestion technique. Ehrlich's haematoxylin and eosin. × 50.

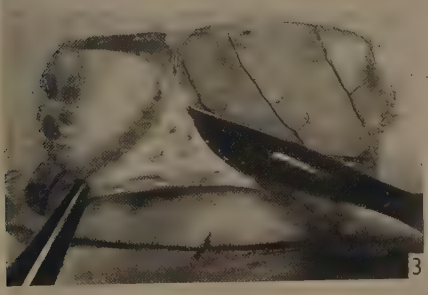
Fig. 21. A 'fitted' pinch graft: mouse's thigh skin transplanted to the chest and so orientated that the hairs point downwards.

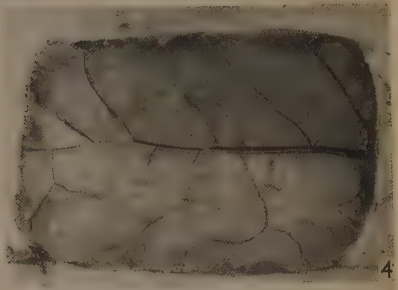
Fig. 22. A graft similar to that illustrated by fig. 21 showing a hair crop of graft specific colour, density and orientation.

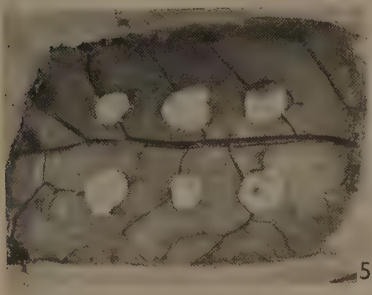
Fig. 23. Showing a group of eight skin homografts (fitted 'pinch' grafts) and one autograft 12 days after transplanation to a rabbit's chest. Breakdown of the homografts is now complete and they appear as hard, dry, reddish brown scabs, whereas the autograft is perfectly normal.

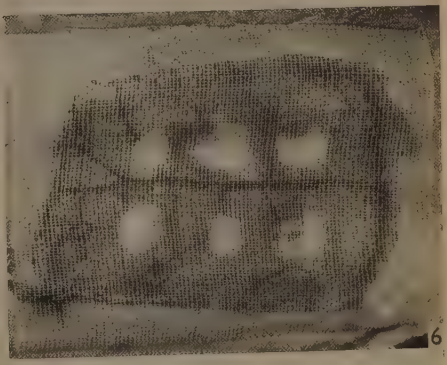

1

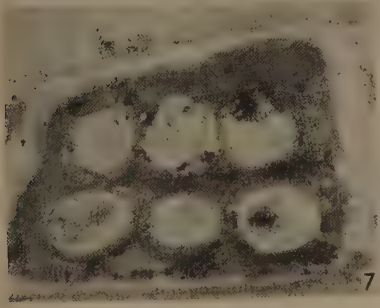

2


3


4


5

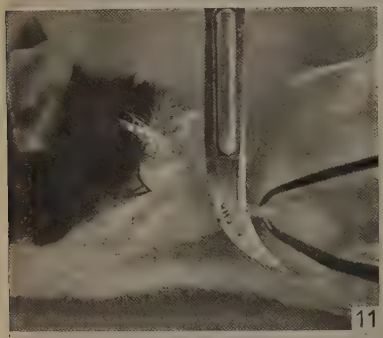

6

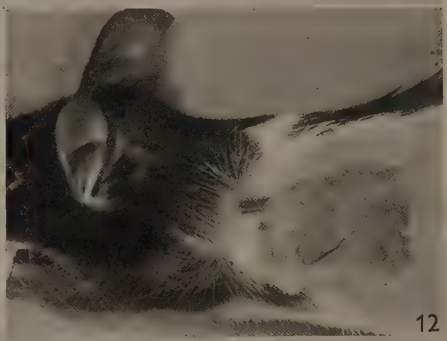

7

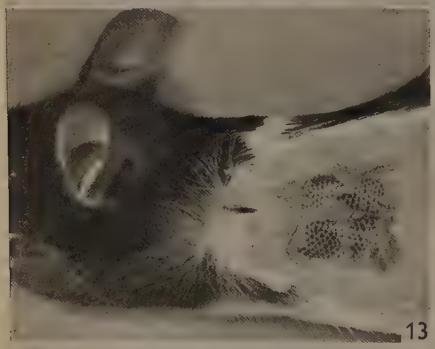

8

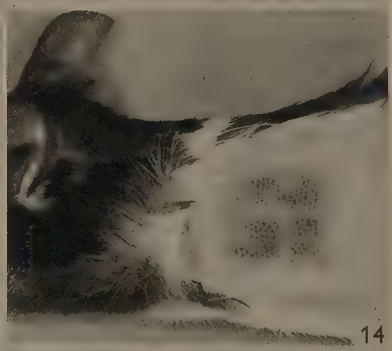

9


10

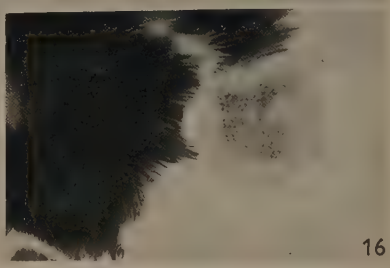

11

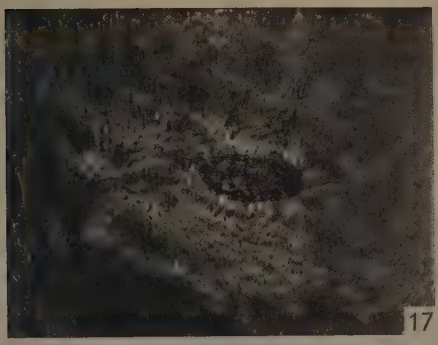

12

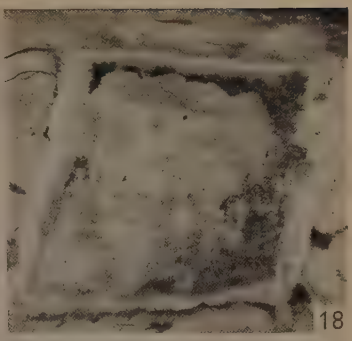

13


14

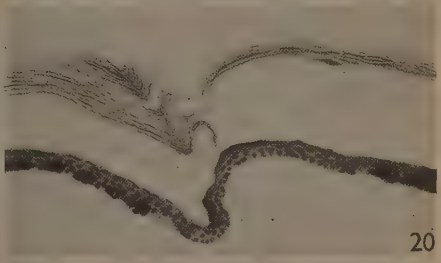

15


16


17

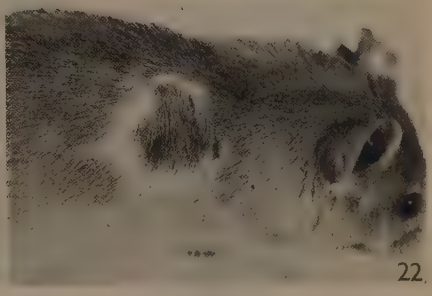

18


19


20


21


23


22

THE FERTILIZATION REACTION IN THE SEA-URCHIN

THE PROBABILITY OF A SUCCESSFUL SPERM-EGG COLLISION

By LORD ROTHSCHILD and M. M. SWANN

From the Department of Zoology, University of Cambridge, and the Marine Station, Millport

(*Received 7 February* 1951)

(With One Text-figure)

INTRODUCTION

The change in cortical structure which passes over the egg surface of *Psammechinus miliaris* in about 20 sec. at 18° C. can only be the block to polyspermy if the probability of a successful sperm-egg collision is lower than one. For during the passage of the cortical change over the egg surface, previous calculations (Rothschild & Swann, 1949) indicate that there will be some fifteen potentially successful sperm-egg collisions after the cortical change has started in an egg immersed in a sperm suspension of density 10^6/ml. An obvious extension of these experiments was to investigate the effect of polyspermy-inducers on the conduction velocity of the cortical change and on the speeds of the spermatozoa. A decrease in the former or an increase in the latter would increase the probability of polyspermy. As, however, nicotine, an excellent polyspermy-inducer in the sea-urchin egg, had neither of these effects, the question again arose of the probability of a sperm-egg collision being low and of nicotine exerting its effect by increasing this probability (Rothschild & Swann, 1950). The methods used to investigate the questions of the low probability of a successful sperm-egg collision and of the relationship, if any, between the cortical change and the block to polyspermy were at that time indirect. Both these questions have now been examined in a more direct way than before. A brief note on the relationship between the conduction velocities of the block to polyspermy and of the cortical change has been published (Rothschild & Swann, 1951), but will be discussed in more detail in this paper. The probability of a successful sperm-egg collision was studied by immersing unfertilized eggs in sperm suspensions of known density for known but varying periods of time, ranging from 0 to 300 sec., and later counting the proportions of fertilized and unfertilized eggs. Interpretation of the results in terms of probabilities involves the hypothesis that a suspension of sea-urchin spermatozoa can be considered analytically as an assemblage of gas molecules, moving in random directions. Apart from the difficulty of verifying this hypothesis experimentally, there are obvious differences between a suspension of spermatozoa and an assemblage of gas molecules. The most serious of these concerns the statement, often made but not substantiated, that a substance diffuses out of unfertilized eggs and has a chemotactic effect on spermatozoa of the same species.

Although chemotaxis of plant spermatozoa towards secretions of plant eggs or archegonia undoubtedly occurs, the position is far less clear in the gametes of animals. Possible explanations, other than chemotaxis in the true sense, of the accumulation of spermatozoa in the neighbourhood of egg secretions and eggs, have been discussed in detail by Rothschild (1951), while we have noted that the dark ground 'tracks' of sea-urchin spermatozoa (Rothschild & Swann, 1949) do not bend or point preferentially towards eggs. Moreover, neither in the case of *Echinus esculentus* nor of *P. miliaris* have we been able to confirm Dakin & Fordham's experiments (1924), in which sea-urchin spermatozoa accumulated to a marked degree in capillaries containing egg water, though Lillie's activation* test for the presence of egg water (1919) was positive. Another possible source of error in the kinetic treatment concerns the effect of egg jelly on spermatozoa. Though many experiments on fertilization are done on eggs without jelly, the jelly may of course be present in natural fertilization. The influence of the jelly has therefore been investigated, parallel experiments being done on eggs with and without jelly. A convenient method of establishing whether jelly is, or is not, present round eggs is to place a sample of the experimental suspension in sea water containing Janus Green B, which is strongly taken up at the surface of the jelly (see Harvey, 1941).

A further difficulty in the interpretation of the experiments centres round the well-known fact that the fertilizing power of a spermatozoon quickly declines with time (Lillie, 1915). Therefore, although eggs were on occasions left in contact with spermatozoa for as long as 300 sec., this was done with the knowledge that interpretation of data obtained after eggs had been in contact with sperm suspensions for minutes rather than seconds might involve new variables which would certainly obscure estimates of the true probability of a successful collision. An arbitrary upper limit of 45 sec. was therefore selected for the time of contact between eggs and spermatozoa. This question is discussed in more detail later. Though of uncertain value for probability estimates, the information gained in the range 45–300 sec. is of interest from another point of view, the decline in the fertilizing capacity of a spermatozoon.

Another source of error is introduced if the sperm suspensions are too dense. The disturbing factor might be called sperm-sperm interactions, and although on *a priori* grounds it might have been expected that in a dense suspension, spermatozoa would 'interfere' with each other, the existence of such interactions at high sperm densities is strikingly demonstrated in the experiments described in this paper. This fact has interesting implications in other spheres of gametological research.

Although the experiments required no apparatus apart from beakers, pipettes and a microscope, the actual procedure was rather complicated, and is therefore described in detail in the next section. A serious, and to a certain extent unexpected, difficulty was experienced in finding a method of suddenly killing or inactivating the spermatozoa, without preventing the eggs developing or exhibiting the normal signs of having been fertilized, such as fertilization membranes, cleavage, or the well-known

* Activation here means the *initiation* of sperm activity, not an increase in activity, in sperm suspensions made motionless by immersion in isotonic NaCl.

differences between the cytolysis of fertilized and unfertilized eggs. The following reagents were tried and found to be unsatisfactory. $CuCl_2 2H_2O$: this inhibits fertilization but only after the eggs have been in sea water containing it for much longer than the duration of these experiments. Acidified sea water: this method of inhibiting fertilization has been used successfully by Tyler & Schultz (1932) and Tyler & Scheer (1937). Using the eggs and spermatozoa of *P. miliaris*, Tyler's results could not be reproduced, no doubt because of species differences. $HgCl_2$: as is well known, this substance is highly toxic to spermatozoa; but it also has an irreversible toxic effect on eggs. The same applied to ceric sulphate and aluminium hydroxide, both of which are toxic to sea-urchin spermatozoa. It was found impossible to arrange the dosage of these substances so that though the spermatozoa were inactivated, the eggs were left in an identifiably fertilized condition. As will be seen, the experimental procedure made it essential that after the desired number of sperm-egg collisions, the spermatozoa should be incapable of recovering from the 'killing' treatment. A suitable concentration of hypotonic sea water (45% sea water in distilled water), however, was found to kill the spermatozoa and to prevent fertilization; but it had no harmful effect on the eggs.

EXPERIMENTAL PROCEDURE

This is conveniently explained with the aid of Table 1, which represents one set (no. 4) of operations in a series, which may contain as many as ten sets, carried out at approximately the same time. A case where the eggs were in contact with a sperm suspension of known density for 5 sec. has been selected. The times on the right in

Table 1. *Experimental procedure*

Series 4	Contents at $t=0$	Seconds
Beaker 1	450 ml. hypertonic sea water	
Beaker 2	90 ml. hypotonic sea water	$t=120$
Beaker 3	10 ml. eggs in sea water	$t=5$
Pipette	0·1 ml. sperm in sea water	$t=0$

Table 1 show when pipettes or beakers were emptied into other beakers. For example, the eggs in beaker 3 were inseminated from the pipette at $t=0$ and after 5 sec. were transferred to beaker 2. The hypotonic sea water in beaker 2 consisted of 35 ml. sea water and 55 ml. distilled water, so that after addition of beaker 3, the contents consisted of 45% sea water in distilled water. The sea water in beaker 1 was made hypertonic with NaCl, the hypertonicity being adjusted so that after addition of the contents of beaker 2, the final 'sea water', in which the eggs were to develop, had the same tonicity as sea water. The success of this experimental procedure depended on the following considerations. (1) As one experiment involved a number of separate operations, each of the type shown in Table 1, consideration had to be given to the possibility that the mean speeds of the different sperm suspensions might vary because of delays during the experiment. This

difficulty was obviated by carrying out the inseminations of the experimental (not the control) suspensions at 15 sec. intervals, in which time the speeds of the spermatozoa in the different suspensions did not decline significantly. This necessitated two people carrying out the operations on a rather complicated time schedule, because after insemination of eggs in beaker 3 in Table 1, for example, these eggs had to be transferred at specified time intervals to two different solutions. Without careful timing there would therefore have been a tendency for the third operation in series 4 to conflict with other operations in different series. At the same time, all the $t=0$ operations had to be carried out as quickly as possible, for the reasons mentioned above. (2) Spermatozoa in beaker 3 must be instantly inactivated on being put into beaker 2, i.e. there must be no possibility of fertilization in beaker 2. Alternatively, the probability of fertilization in beaker 2 must be determined. This contingency can be dealt with by a control in which eggs and spermatozoa are added simultaneously to beaker 2, run at the same time as the main experiment. (3) Spermatozoa which appear to have been killed by the hypotonic sea water in beaker 2 may recover after transfer to beaker 1. This possibility can also be dealt with by a control experiment in which unfertilized eggs are added to beaker 1, spermatozoa to beaker 2, and the contents of beaker 2 to beaker 1, after 120 sec. (4) Eggs may be parthenogenetically activated by the hypotonic-hypertonic sea-water treatment. This eventuality necessitates a further control in which all operations are normal but spermatozoa are omitted from the series. (5) Another control is needed to determine the percentage fertilization at the same sperm and egg density as in the experimental series, in a sample from the same egg suspension, under normal conditions.

Before each operation in each series, beakers were very gently agitated by hand, in an irregular way. The experiments, on eggs of *P. miliaris* with and without jelly, were done at room temperature which varied from 17 to 18·5° C. during the season.

Sperm counts were made absorptiometrically and, as the new model of the Spekker was used, the procedure was slightly different from that previously described. In the new model there is a linear drum scale and 'zero' is 1·30 instead of 2·00 as in the earlier model. Instructions to cover such differences have been published (Rothschild, 1950).

RESULTS

Eggs and spermatozoa were allowed to interact for a series of known but different times; later, counts were made of the number of fertilized and unfertilized eggs in samples corresponding to each interaction time. The resultant information, which is depicted graphically in Fig. 1 *a*, *b*, enables an estimate to be made of the probability of a successful sperm-egg collision, p,* or of the number of sperm-egg collisions that on the average will be needed for fertilization to take place. The method of making these estimates is given in an Appendix at the end of the paper. The results of all experiments, at different sperm densities, are given in Table 2, from which it will be seen that the probability of a successful sperm-egg collision varies with the

* Except in a few self-evident cases, p is an *estimate* of the actual probability, often written $\hat{p}$.

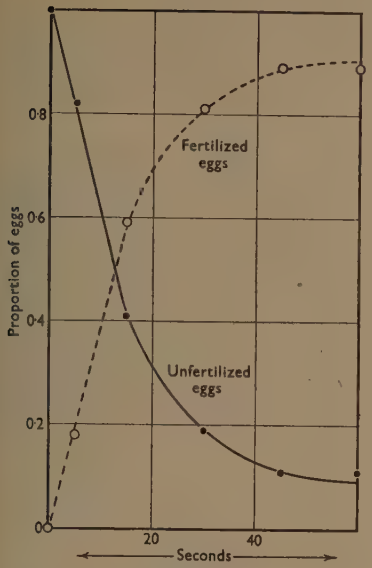


Fig. 1 (*a*).

Fig. 1*a*. Proportions of fertilized and unfertilized eggs after various times of contact between unfertilized eggs and spermatozoa (density, $3{\cdot}67 \times 10^5$/ml.) of *Psammechinus miliaris*.

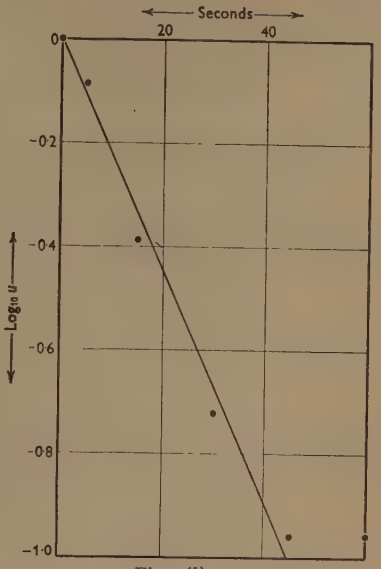


Fig. 1 (*b*).

Fig. 1*b*. The same experiment as in Fig. 1*a*, plotted semi-logarithmically. This is required for the probability calculations. *u*, proportion of unfertilized eggs.

Table 2. *Variation in the average number of sperm-egg collisions necessary for fertilization, N, or in the estimated probability of a successful collision, p, with sperm density, n*

n	*N*	*p*	*I*
$7{\cdot}44 \times 10^4$	4·43	0·226	0·263, 0·189
$9{\cdot}84 \times 10^4$	8·27	0·121	0·144, 0·098
$1{\cdot}59 \times 10^5$	11·63	0·086	0·103, 0·069
$2{\cdot}11 \times 10^5$	6·58	0·152	0·180, 0·124
$2{\cdot}53 \times 10^5$	7·04	0·142	0·183, 0·101
$2{\cdot}89 \times 10^5$	10·10	0·099	0·128, 0·070
$3{\cdot}09 \times 10^5$	8·33	0·120	0·139, 0·101
$3{\cdot}67 \times 10^5$	8·70	0·115	0·132, 0·098
$7{\cdot}50 \times 10^5$	13·70	0·073	0·084, 0·062
$8{\cdot}29 \times 10^5$	14·71	0·068	0·078, 0·058
$1{\cdot}97 \times 10^6$	24·39	0·041	0·047, 0·035
$2{\cdot}99 \times 10^6$	31·25	0·032	0·037, 0·027
$3{\cdot}43 \times 10^6$	41·67	0·024	0·028, 0·020
$5{\cdot}11 \times 10^6$	50·00	0·020	0·023, 0·017
$7{\cdot}50 \times 10^6$	142·86	0·007	0·008, 0·006
$9{\cdot}18 \times 10^6$	66·67	0·015	0·017, 0·012
$9{\cdot}62 \times 10^6$	90·91	0·011	0·013, 0·009

I, interval within which *p* lies (fiducial probability 0·95).

number of spermatozoa in the sea water round the eggs. At a sperm density of about 10^7/ml., by no means a dense suspension, the probability of a successful collision is 1·5 in 100. This sperm density, which comes into the category of 'very dilute' (Rothschild, 1951), would be too low for use in a standard manometric experiment.

Effect of jelly on p. The technique of subjecting eggs to a known number of sperm-egg collisions is probably the best method of quantitatively investigating the effect of varying environmental conditions on the fertilization reaction. One of the most interesting of these environmental conditions concerns the jelly which in nature surrounds the eggs, but which is removed in most fertilization experiments. The effect of jelly on p is shown in Table 3, from which it will be seen that its presence increases the probability of a successful collision. In the case of Exp. 1 in this table, reference to Table 2 shows that even if a scaling factor were introduced for the decline in p with increasing sperm density, which is trivial over such a small range, the removal of the jelly still reduces the probability of fertilization. This question does not arise in the other experiments in this series, as the sperm density was the same in the presence and absence of jelly.

Table 3. *Influence of egg jelly on the fertilization reaction. The same eggs and spermatozoa were used in each pair of experiments*

Exp. no.	Jelly	Sperm density	p	Standard error of p
1	Present	$3{\cdot}17 \times 10^5$	0·177	0·013
	Absent	$3{\cdot}67 \times 10^5$	0·122	0·009
2	Present	$2{\cdot}53 \times 10^5$	0·182	0·023
	Absent	$2{\cdot}53 \times 10^5$	0·142	0·021
3	Present	$2{\cdot}89 \times 10^5$	0·169	0·022
	Absent	$2{\cdot}89 \times 10^5$	0·099	0·015

Block to polyspermy. The experiments described in this paper led logically on to an examination of the conduction velocity of the block to polyspermy. Although a brief note on these experiments, which were of a preliminary nature, has been published (Rothschild & Swann, 1951), it is convenient to recapitulate the results as they are so closely bound up with the others reported in this paper. The results of one experiment are given in Table 4. The experimental procedure was to run two experiments at the same time: in Exp. 1 the spermatozoa were killed in the usual way after a known time, 25 sec.; in Exp. 2, at the time when the spermatozoa were killed in Exp. 1, more spermatozoa were added, the sperm density being increased by a factor of 100. From results described earlier in this paper, it is known what proportion of eggs will have been fertilized in a particular time at a particular sperm density and therefore how many blocks to polyspermy will have started during that time. If, therefore, instead of killing the spermatozoa at $t = 25$, the intensity of the sperm bombardment is increased, there should be a negligible incidence of polyspermy unless a considerable number of sperm-egg collisions occur during the propagation of the block to polyspermy.

Table 4 shows that there are some three times as many polyspermic eggs in Exp. 2 as there were unfertilized eggs in Exp. 1. In other words, nearly half of the eggs which were fertilized in 25 sec. had not finished propagating their block to polyspermy in that time, and became polyspermic because of the new sperm bombardment they received after the 25 sec. period was terminated.

Table 4. *Conduction velocity of block to polyspermy*

Time, sec.	Exp. 1	Exp. 2
0	Sperm added (3×10^6/ml.)	Sperm added (3×10^6/ml.)
25	Sperm killed	More sperm added (3×10^8/ml.)
Unfertilized, %	13	2
Monospermic, %	85	54
Polyspermic, %	2	44

DISCUSSION

Sources of error: $\bar{c}$;. Some of the sources of error, associated with treating a suspension of spermatozoa as an assemblage of particles or gas molecules moving in random directions, have been mentioned earlier (pp. 403, 404). A further source of error may be introduced by the assumption that the mean speed of a sperm suspension, $\bar{c}$, is 150 μ/sec. This requires two comments. First, it is a lower figure than that previously given (180 μ/sec., Rothschild & Swann, 1949). Dark ground sperm tracks taken during the course of these experiments indicated that the lower figure was the more accurate, at any rate during the season in question.* Secondly, the figure 150 μ/sec. is assumed not to vary with sperm density. The variation of a spermatozoon's speed according to the density of the suspension is a complicated subject. Earlier views that sperm speeds were inversely proportional to density were mainly based on measurement of O_2 uptake which is a poor index of motility (Rothschild, 1948). In any case, no significant differences between sperm speeds in dense and dilute suspensions were observed using the dark ground track system (Rothschild & Swann, 1950), an observation which was confirmed at various sperm densities this year. The existing evidence therefore supports the view that sperm speeds do not vary greatly with density. Superficially, the fact that the probability of a successful collision declines with increasing sperm density might be held to lend support to the view that sperm speeds are lower in dense suspension. This, however, is an unlikely explanation of the decline in p at the higher densities because the sperm speeds would have to be so low to account for the observed probabilities, which differ by a factor of 20 when sperm densities differ by a factor of 100. The variation in p may be due to sperm-sperm interactions of a mechanical nature.

Sources of error: slope of the $\log_{10} u$, t plot. The arbitrary selection of the period 0–45 sec. (Fig. 1*b*) for the time during which it is safe to assume that the speed or fertilizing capacity of a spermatozoon remains constant may introduce errors. In future experiments, for example on the influence of changes in environmental

* We are indebted to Mrs M. M. Swann for taking the dark ground sperm track photographs.

conditions on the fertilization reaction, it would be safer to restrict observations to a relatively short period, as was done in the experiments on the effect of jelly on p. The statistical analysis supports this contention. In all experiments the values of u for t large, 45–300 sec., were lower than would have been expected on the basis of the slope of the $\log_{10} u, t$ plot up to $t = 45$. This deviation from linearity, which is very clear in Fig. 1*b*, is a measure of the decline in the fertilizing capacity of the spermatozoa, either through a decrease in speed, or through the loss of some substance from the spermatozoa to the medium, with concomitant impairment of fertilizing capacity.

Sources of error: n. Estimates of n, the sperm density, may introduce a rather serious source of error, not because the actual counts are subject to large error—the fiducial limits are of course known (Rothschild, 1950); but because there is at present no way of counting the proportions of live and dead spermatozoa in a suspension. This problem is well known in the field of mammalian sperm physiology, in which it has been solved (Hancock, 1951).

Egg jelly. One possible explanation of the beneficial action of egg jelly is that it acts as a sperm trap (Rothschild & Swann, 1949). When eggs are inseminated with fairly dense sperm suspensions, more spermatozoa are observed in the neighbourhood of an egg than in an equivalent volume of nearby sea water. This happens because spermatozoa which collide accidentally with the jelly tend to stick to it, while, on the average, the same number of spermatozoa enter and leave an ordinary volume element of sea water. It does not follow that because an abnormal number of spermatozoa stick to the surface of the jelly, an abnormal number of spermatozoa will collide with the egg surface. The surface of the jelly presents a distinct barrier to the passage of spermatozoa, as can be seen by comparing the number of spermatozoa within the jelly, which is quite fluid, with the number on the jelly surface.* The claim has sometimes been made that spermatozoa sustain a *loss* in fertilizing capacity after being in contact with egg jelly in sea water. There are reasons for querying the occurrence of this phenomenon under natural conditions (Rothschild, 1951) and these experiments show that even if it occurs, it has no bearing on the fertilization reaction in nature. Two other possible explanations of the effect of jelly in increasing p are worthy of mention. First, jelly may increase the mean speed of the suspensions, thus increasing the egg-bombardment frequency. There is no evidence for or against this explanation. Secondly, the jelly may have some orienting effect on the spermatozoa, increasing the probability that they collide with the egg surface at the right angle (Tyler, 1948). There is no evidence for or against this explanation, though the possibility that egg jelly has a function of this type has attracted the attention of a number of workers. Perhaps the most likely explanation is that egg jelly has a beneficial effect on spermatozoa in preventing loss of fertilizing capacity, which is equivalent to increasing the sperm density.

The block to polyspermy. The experiments on this subject suffer from the disadvantage that a sperm density of 3×10^8/ml. is too high to obtain a morphologically

* The argument that the jelly merely increases the effective egg radius, and therefore the bombardment frequency, is open to similar objections.

satisfactory polyspermic fertilization reaction. At such high sperm densities there is a tendency for eggs to cytolyse, which may be due to an excessive number of spermatozoa entering the eggs; but it may also be connected with a high concentration of the haemolytic substance Androgamone III in the neighbourhood of the eggs. When counting eggs at the end of the experiment, a decision, which on occasions is difficult, has to be made as to whether an egg should be considered as cytolysed or polyspermic. As under normal conditions of insemination there is a negligible percentage of cytolysed eggs, it might be held that all eggs which are cytolysed under conditions of insemination with abnormally high sperm densities are cytolysed because of polyspermy. If this problem were obviated by reducing the sperm density resulting from the second insemination, the number of polyspermic eggs would be reduced and the results of the experiment would be correspondingly less clear cut. When these experiments are repeated more systematically, a series at lower second insemination sperm densities will be carried out.

In order to obtain some quantitative information regarding the conduction velocity of the block to polyspermy, as revealed by this experiment, the incidence of polyspermic eggs may be examined on the assumption that the block to polyspermy takes 1 or 20 sec. to pass completely over the egg surface. If eggs and spermatozoa are left in contact with each other for 24 sec., at a sperm density of 3×10^6/ml., the percentage of fertilized eggs at the end of that time will be about 85. One second later, that is 25 sec. after the beginning of the experiment, none of these fertilized eggs will be available for polyspermy. The maximum percentage of polyspermic eggs, if the conduction time of the block to polyspermy is 1 sec., is therefore 15. This is incompatible with the results in Table 4, in which the percentage of polyspermic eggs was 44, following the addition of more spermatozoa at $t=25$. If, however, the conduction time of the block to polyspermy is 20 sec., which is also the conduction time of the cortical change, the position is different. Five seconds after the beginning of the experiment, 45% of the eggs will still be unfertilized. These eggs will not have finished propagating their blocks to polyspermy by the time the additional spermatozoa are added at $t=25$, and consequently a little less than 45% of the eggs in the suspension will be available for polyspermy. The observed percentage of polyspermic eggs was 44. When this experiment has been systematically repeated, it may be possible to make a more accurate estimate of the conduction time of the block to polyspermy. Although this experiment is inconsistent with the concept of a high-speed block to polyspermy, with or without a high probability of a successful collision, interpretation on the basis of a 20 sec. block to polyspermy introduces difficulties which cannot be resolved without further experiments. Suppose that in a particular suspension, 80% of the eggs are fertilized in 20 sec. This means that each of these eggs has sustained an effective collision in that 20 sec. In the next 20 sec. a number of eggs in this 80% will receive further effective collisions. The number of such collisions will be of the order of 0·7 at the sperm density in question. But the proportion of polyspermic eggs at this sperm density is far lower than this. The implication of this argument is that though it has been proved that the conduction time of the block to polyspermy is longer than has

hitherto been suspected (i.e. more than 1 sec.), and that there is an associated low probability of a successful collision, the cortical change cannot be equated to the block to polyspermy without making additional hypotheses which at present are not experimentally justified.

General. As might be expected, the development of a new method of investigating the fertilization reaction tends to raise new questions rather than answer old ones. On the basis of previous work, it was predicted that the probability of a successful sperm-egg collision was significantly less than one: this prediction has now been verified; but new problems, such as the decline in the probability of a successful collision with increasing sperm density, have arisen. Possible explanations of this phenomenon have been put forward, but further experiments are required before they can be confirmed or rejected. The principle of subjecting eggs to known concentrations of spermatozoa for known periods of time suggests a series of experiments involving the effects of variations in the environment on the fertilization reaction. For example, a number of agents are known to extend the fertilizing capacity of spermatozoa. But it is not known how this extension is achieved; nor can such effects in general be put on a quantitative basis. Equally, it should now be possible quantitatively to examine the effects of ageing, both of spermatozoa and of eggs, on the fertilization reaction. If such experiments were carried out in the same detail as those described in this paper, they would be extremely time-consuming. But, as is shown in the Appendix, estimates of p can be made when eggs have been subjected to known concentrations of spermatozoa for only two times, for example 10 and 30 sec.

APPENDIX

Estimation of the probability of a successful sperm-egg collision

(1) *Provisional estimate of p.* Determination of the probability of a successful sperm-egg collision involves a knowledge of the number of collisions in unit time, Z, and of the observed proportions of fertilized and unfertilized eggs, f and u, in a suspension which has been in contact for time t with spermatozoa at density n.

Consider a typical egg in the egg population. On the average, one spermatozoon will collide with this egg every $\tau\ (=1/Z)$ sec. This is equivalent to a series of successive trials, at times τ, 2τ, 3τ,..., there being a constant probability p of a success being associated with each trial. In this case a trial is a collision and a success is a successful collision. The probability P_r of it taking exactly r trials to achieve a successful collision is equal to the probability that the first $(r-1)$ trials will be unsuccessful and the rth trial successful, i.e.

$$P_r = q^{r-1}p, \tag{1}$$

where $q = 1-p$.

By the Addition Theorem, the probability of a successful trial (a collision followed by fertilization), at any one of the times τ, 2τ, 3τ,..., $s\tau$, is given by

$$P = \sum_{1}^{s} q^{r-1}p \tag{2}$$

$$= 1 - e^{-\alpha t}, \tag{2·1}$$

where $\alpha = -(\log q)/\tau$ and $\log q$ is negative. In terms of a sample from the population of eggs,

$$f = 1 - e^{-\alpha t}, \tag{3}$$

$$u = e^{-\alpha t}. \tag{3·1}$$

Equation (3·1) can be written in the form

$$\log u = \frac{\log q}{\tau} t, \tag{3·2}$$

so that if $\log u$ is plotted against t, the slope of this line, α, is given by $(\log q)/\tau$. As α can be measured, $\log q$, which equals $-\tau\alpha$, can be evaluated: p, the probability of a successful collision, equals $1 - q$. Calculations relevant to one particular experiment are given in Table 5, in which the number of fertilized and unfertilized eggs found in egg suspensions which had been in contact for different times with sperm suspensions of known density is shown. Immediately below this is given the proportion of unfertilized eggs corresponding to the actual numbers, but corrected for the incidence of fertilization (1%), which occurred when eggs and spermatozoa were in contact with each other for 0 sec. No correction was necessary for the incidence of unfertilizable eggs in this example, and in other cases these two corrections never amounted to more than 3%. The calculations for the provisional estimate of p follow. The slope of the $\log_{10} u$, t plot up to 45 sec. (Fig. 1*b*) was determined by minimizing the sums of squares.

Table 5. *Procedure for making a provisional estimate of p*

Time of contact, t, between eggs and sperm, in sec.	—	0	5	15	30	45	60	300
No. of fertilized eggs	60	1	14	43	58	80	72	68
No. of unfertilized eggs	0	71	59	30	13	10	9	0
Scaled proportion of unfertilized eggs, u	—	1·00	0·82	0·41	0·19	0·11	0·11	0·00
$\text{Log}_{10} u$	—	0	−0·0862	−0·3872	−0·7212	−0·9586	−0·9586	$-\infty$

Slope (α) of $\log_{10} u$, t ($t = 0-45$), $= -\frac{71 \cdot 012}{3175} = -0 \cdot 0224$.

Sperm density, $3 \cdot 67 \times 10^5$/ml.

$\tau = 2 \cdot 3129$.

Log $q = \tau\alpha = -0 \cdot 0517$.

$q = 0 \cdot 888$.

$p = 0 \cdot 11$

Z, the number of collisions in unit time, is a function of the egg radius a, the sperm density n, and the mean speed of the sperm suspension $\bar{c}$. The relationship $Z = \pi a^2 n \bar{c}$ has been given and discussed in previous papers (Rothschild & Swann, 1949).

(2) *Accurate estimate, p.* If, at time t_i, f_i fertilized and u_i unfertilized eggs have been counted in a sample of N_i eggs, the probability of obtaining such a result in random sampling is given by

$$L = \binom{N_i}{f_i} Q_i^{u_i} (1 - Q_i)^f, \tag{4}$$

where $Q_i = 1 - P_i$ = the true proportion of unfertilized eggs after time t. Since the results obtained in one sample do not affect the results in other samples, the probability of obtaining the combined data is

$$L = \prod_1^k L_i = \prod_1^k \binom{N_i}{f_i} Q_i^{u_i} (1 - Q_i)^{f_i}. \tag{5}$$

The data is made up of k samples at times t_i, $i = 1 \ldots k$. L is a function both of the data and of the parameter α, contained in the probabilities Q_i. When L is regarded as a function only of the data, it gives the probability of observing each possible combination of the data; but when considered as a function of α, the data being given, it is known as the Likelihood of each possible value of α (Fisher, 1922). The operation of maximizing the Likelihood enables a choice to be made of the α which is most appropriate to the data. In many cases the logarithm of the Likelihood, rather than the Likelihood itself, is used for finding the maximum. This is permissible since $\log L$ is a steadily increasing function of L. For L or $\log L$ to be a maximum

$$\begin{aligned} -\frac{\partial}{\partial \alpha} \log L &= \sum_1^k \left(\frac{u_i}{Q_i} - \frac{f_i}{1 - Q_i} \right) t_i Q_i \\ &= \sum_1^k \left(u_i - f_i \frac{Q_i}{1 - Q_i} \right) t_i \\ &= 0. \end{aligned} \tag{6}$$

Table 6. *Procedure for estimating α by Maximum Likelihood Method*

t	$Q = e^{-\alpha t}$		f	u	Score, $K = (u - fQ/P)\,t$		$I(\alpha)$	$I(\alpha)/N$	$K^2/I(\alpha)$
	α_1	α_2			α_1	α_2			
5	0·7727	0·7681	14	59	+57·035	+63·145	6,045	83	0·660
15	0·4613	0·4531	43	30	−102·330	−84·375	13,608	186	0·523
30	0·2129	0·2053	58	13	−80·640	−59·520	16,508	233	0·215
45	0·0982	0·0930	80	10	+57·960	+80.865	18,687	208	0·350
Note: α_1 = provisional α; α_2 = final α				Σ	−67·975	+0·115	54,848		1·748

The expression $-\partial/\partial\alpha \log L$ is called the Score and finding the Maximum Likelihood estimate is equivalent to setting the Score equal to zero. This method of analysing the data is shown in Table 6. The plot of $\log (u/N)$ against t was found to give an approximately straight line (Fig. 1*b*, p. 5) with slope 0·0224. This provides a provisional estimate of α, $\log 10 \times 0{\cdot}0224 = 0{\cdot}0516$. Examination of the total Score, −67·975, shows that this provisional estimate of α is too low. Further trials and linear interpolation show that if $\alpha = 0{\cdot}052775$, the total Score becomes +0·115, which is sufficiently near zero for our purposes. A difference ΔK from a zero Score causes a systematic error $\Delta\alpha$ from the true estimate of α. $\Delta\alpha/S(\alpha) = \Delta K/\sqrt{[I(\alpha)]}$. If this latter quantity is numerically less than 0·1, $\Delta\alpha$ is small compared with the sampling error of α. As $p = 1 - \exp(-\alpha\tau)$, $p = 0{\cdot}115$.

(3) *Precision of p.* The sampling variance of the Maximum Likelihood estimate is given by

$$1/V(p) = -\mathscr{E}\left\{\left(\frac{\partial^2 \log L}{\partial p^2}\right)\right\} = \mathscr{E}\left\{\left(\frac{\partial \log L}{\partial p}\right)^2\right\} = I(p), \tag{7}$$

where $\mathscr{E}(\lambda)$ = the average value of λ, and $I(p)$ = the amount of information associated with the estimate. Similarly, it can be shown that

$$I(\alpha) = \sum_{1}^{k} \left\{ N_i t_i^2 \left(\frac{Q_i}{1 - Q_i} \right) \right\}, \tag{8}$$

$I(p)$ and $I(\alpha)$ being related by the equation

$$I(p)/I(\alpha) = \tau^{-2} e^{2\alpha\tau}. \tag{9}$$

From these relationships it is found (approximately) that the variance of p is

$$V(p) = 0{\cdot}7639 \times 10^{-4}.$$

The standard error of the estimate of p is

$$S(p) = 8{\cdot}7402 \times 10^{-3}.$$

There is therefore a fiducial probability of 0·95 that p, the estimate of which is 0·115, lies between 0·132 and 0·098.

(4) *Initial hypothesis.* The underlying hypothesis is that after the addition of spermatozoa, the proportion of unfertilized eggs declines exponentially with time. $K/\sqrt{[I(\alpha)]}$ is distributed as a normal variate with zero mean and unit variance for each sample; consequently $\Sigma\{K^2/I(\alpha)\}$ is distributed as χ^2, the number of degrees of freedom being three, not four, since p has been used in the determination of these quantities. The values of $K^2/I(\alpha)$ and $\Sigma\{K^2/I(\alpha)\}$ are given in Table 6, the latter being 1·748. Such a value of χ^2 on three degrees of freedom is attained or exceeded with a probability of slightly less than 0·70, which shows that there are insufficient grounds for rejecting the original hypothesis.

(5) *'Best' interaction times.* Examination of Table 6 shows that $I(\alpha)/N$, the amount of information per egg, is markedly lower at 5 sec. than at the other times during which the eggs and spermatozoa were allowed to interact, and is a maximum at 30 sec. The importance of being able to assess the 'best' period for interaction, in reducing the time-consuming nature of these experiments, needs no emphasis.

SUMMARY

1. Unfertilized eggs of the sea-urchin (*Psammechinus miliaris*) were left for known but varying times in contact with homologous sperm suspensions containing known numbers of spermatozoa. Counts were made of the numbers of fertilized and unfertilized eggs at times ranging from 0 to 300 sec. after mixing.

2. If spermatozoa are considered as particles moving in random directions, the frequency of sperm-egg collisions can be calculated if the density and mean speed of the sperm suspension are known.

3. The information in (1) and (2) enables an estimate to be made of the probability of a successful sperm-egg collision.

4. The estimated probability of a successful collision, p, varies with sperm density, d. At the lowest density used, $7{\cdot}44 \times 10^4$/ml., p was found to be 0·226. At the highest density, $9{\cdot}62 \times 10^6$/ml., p was about 0·011. The inverse relationship between p and d may be due to sperm-sperm interactions of a physical nature.

5. The presence of jelly round the eggs increases p. This disposes of the possibility, raised in the past, that egg jelly may have an adverse effect on the fertilizing capacity of homologous spermatozoa under normal conditions of fertilization.

6. The technique of subjecting eggs to a pre-determined number of collisions facilitates investigation of the conduction time of the block to polyspermy. Preliminary experiments suggest that the conduction time may be of the order of seconds rather than fractions of a second.

7. Sources of error arising from the 'kinetic' treatment of sperm suspensions are discussed in detail. One source of error concerns the alleged chemotaxis of spermatozoa towards eggs and egg secretions. No chemotaxis was observed.

We are greatly indebted to Prof. R. A. Fisher, F.R.S., for patient and unstinting advice.

REFERENCES

DAKIN, W. J. & FORDHAM, M. G. C. (1924). The chemotaxis of spermatozoa and its questioned occurrence in the animal kingdom. *J. exp. Biol.* **1**, 183.

FISHER, R. A. (1922). On the mathematical foundations of theoretical statistics. *Philos. Trans. A*, **222**, 309.

HANCOCK, J. L. (1951). A staining technique for the study of temperature-shock in semen. *Nature, Lond.*, **167**, 323.

HARVEY, E. B. (1941). Vital staining of the centrifuged *Arbacia punctulata* egg. *Biol. Bull. Woods Hole*, **81**, 114.

LILLIE, F. R. (1915). The fertilizing power of sperm dilutions of *Arbacia*. *Proc. Nat. Acad. Sci. Wash.*, **1**, 156.

LILLIE, F. R. (1919). *Problems of Fertilization*. University of Chicago Press.

ROTHSCHILD, Lord (1948). The physiology of *Echinus esculentus* spermatozoa. *J. exp. Biol.* **25**, 15.

ROTHSCHILD, Lord (1950). Counting spermatozoa. *J. exp. Biol.* **26**, 388.

ROTHSCHILD, Lord (1951). Sea-urchin spermatozoa. *Biol. Rev.* **26**, 1.

ROTHSCHILD, Lord & SWANN, M. M. (1949). The fertilization reaction in the sea-urchin egg. A propagated response to sperm attachment. *J. exp. Biol.* **26**, 164.

ROTHSCHILD, Lord & SWANN, M. M. (1950). The fertilization reaction in the sea-urchin egg. The effect of nicotine. *J. exp. Biol.* **27**, 400.

ROTHSCHILD, Lord & SWANN, M. M. (1951). The conduction time of the block to polyspermy in the sea-urchin egg. *Exp. Cell. Res.* **2**, 137.

TYLER, A. (1948). Fertilization and immunity. *Physiol. Rev.* **28**, 180.

TYLER, A. & SCHULTZ, J. (1932). Inhibition and reversal of fertilization in the eggs of the echiuroid worm, *Urechis caupo*. *J. exp. Zool.* **63**, 509.

TYLER, A. & SCHEER, B. T. (1937). Inhibition of fertilization in eggs of marine animals by means of acid. *J. exp. Zool.* **75**, 179.